FORM PTO-1390 (REV 5-93)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY DOCKET NO. P564-8019
	TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	DATE: September 15, 1998
		U.S. APPLN. NO. (IF KNOWN, SEE 37 CFR 1.5) 09/147036

INTERNATIONAL APPLICATION NO. PCT/EP96/01130	INTERNATIONAL FILING DATE 15 March 1996	PRIORITY DATE CLAIMED

TITLE OF INVENTION: Jochen MAURER, Joachim JOSE, Thomas MEYER

APPLICANT(S) FOR DO/EO/US: EXPORT SYSTEMS FOR RECOMBINANT PROTEINS

1. XX This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
   (THE BASIC FILING FEE IS ATTACHED)

2. _ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. XX This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT articles 22 and 39(1).

4. XX A proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. _ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. _ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. _ has been transmitted by the International Bureau.
   c. _ is not required, as the application was filed in the United States Receiving Office (RO/US)

6. XX A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. XX Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. XX are transmitted herewith (required only if not transmitted by the International Bureau).
   b. _ have been transmitted by the International Bureau.
   c. _ have not been made; however, the time limit for making such amendments has NOT expired.
   d. _ have not been made and will not be made.

8. XX A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. XX An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)) signed by Jochen MAURER and Joachim JOSE.

10. _ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. _ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. XX An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. XX A FIRST preliminary amendment.
    _ A SECOND or SUBSEQUENT preliminary amendment.

14. _ A substitute specification.

15. _ A change of power of attorney and/or address letter.

16. XX Other items or information: PCT/IPEA/416, PCT/IPEA/409, PCT/ISA/210, PCT/RO/101
    CHECK NO. 17496
    Drawings - 28 sheets

U.S. APPLN. NO. (IF KNOWN, SEE 37 C.F.R. 1.50)	INTERNATIONAL APPLICATION NO. PCT/EP96/00130	ATTORNEY DOCKET NO. P564-8019 DATE: September 15, 1998

17. XX The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)-(5):

Search Report has been prepared by the EPO or JPO.....$930.00
International preliminary examination fee paid to USPTO (37 CFR 1.482)....$720.00
No international preliminary examination fee paid to USPTO (37 CFR1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))....$790.00
Neither international preliminary examination fee (37 CFR 1.482) or international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....$1,070.00
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) .....$ 98.00

	CALCULATIONS	PTO USE ONLY
ENTER APPROPRIATE BASIC FEE AMOUNT =	$930	
Surcharge of $130.00 for furnishing the oath or declaration later than _ 20 _ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$00	

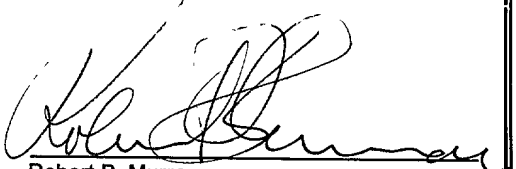
Claims	Number Filed	Number Extra	Rate		
Total Claims	40 - 20 =	20	X $ 22.00	$00	
Independent Claims	04 - 3 =	01	X $ 82.00	$00	
Multiple dependent claim(s) (if applicable)			+ $270.00	$00	
TOTAL OF ABOVE CALCULATIONS =				$930	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$00	
SUBTOTAL =				$930	
Processing fee of $130.00 for furnishing the English translation later the _ 20 _ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$00	
TOTAL NATIONAL FEE =				$930	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$40	
TOTAL FEES ENCLOSED =				$970	
				Amount to be refunded	$
				Charged	$

a. XX A check in the amount of $970 to cover the above fees is enclosed.
b. _ Please charge my Deposit Account No. 14-1060 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.
c. XX The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1060 .

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

NIKAIDO, MARMELSTEIN, MURRAY AND ORAM
Metropolitan Square
655 15th Street, N.W.
Suite 330 - G Street Lobby
Washington, D.C. 20005-5701
Telephone No. (202) 638-5000



Robert B. Murray
Reg. No. 22,980

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Jochen MAURER et al

Serial No.: Unknown

Filed: September 15, 1998

For: EXPORT SYSTEMS FOR RECOMBINANT PROTEINS

PRELIMINARY AMENDMENT

Assistant Commissioner
for Patents
Washington, D.C. 20231

September 15, 1998

Sir:

Prior to calculation of the filing fee and prior to the examination of this application, please amend the above-identified application as follows:

IN THE CLAIMS:

Please amend the claims as follows:

Claims 3, 4 and 5, line 1 of each, delete "or 2".

Claims 9 and 10, line 1 of each, delete "any of Claims 1-8" and insert therefor --claim 1--.

Claim 15, line 1, delete "any of Claims 1-14" and insert therefor --claim 1--.

Claim 17, line 1, delete "any of Claims 1-16" and insert therefor --claim 1--.

Claim 19, line 1, delete "any of Claims 15-18" and insert therefor --claim 15--.

Claim 22, line 1, delete "any of Claims 20-21" and insert therefor --claim 20--.

Claim 23, line 1, delete "any of Claims 20-22" and insert therefor --claim 20--.

Claim 25, line 1, delete "any of Claims 1-24" and insert therefor --claim 1--.

Claim 38, line 1, delete "any of Claims 36-37" and insert therefor --claim 36--.

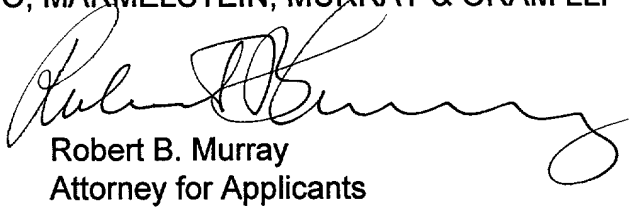
Claim 40, lines 1 and 2, delete "any of Claims 37-39" and insert therefor --claim 37--.

REMARKS

The above amendment to the claims has been made to correct the multiple dependency of the claims and to put the application in better condition for examination.

In the event that any fees are due in connection with this paper, please charge our Deposit Account No. 14-1060.

Respectfully submitted,
NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP



Robert B. Murray
Attorney for Applicants
Reg. No. 22,980

Atty. Docket No.: P564-8019
Metropolitan Square
655 15th Street, N. W.
Suite 330 - G Street Lobby
Washington, D. C. 20005-5701
Tel (202) 638-5000
Fax (202) 638-4810

RBM/cb

Applicant or Patentee: ______
Serial or Patent No.: ______
Filed or Issued: ______
For: Export systems for recombinant proteins

Attorney's Docket No.: 0110-7117 13834P US-WC

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27 (d) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V.
ADDRESS OF ORGANIZATION Hofgartenstraße 2, D-80539 München, Federal Republic of Germany

TYPE OF ORGANIZATION

[ ] UNIVERISITY OR OTHER INSTITUTION OF HIGHER EDUCATION
[ ] TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501 (c) (3))
[ ] NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE ______)
(CITATION OF STATUTE ______)
[x] WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) AND 501(c) (3) IF LOCATED IN THE UNITED STATES OF AMERICA
[ ] WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE ______)
(CITATION OF STATUTE ______)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled Export systems for recombinant proteins by inventor(s) Jochen Maurer, Joachim Jose, Thomas F. Meyer described in

[ ] the specification filed herewith
[XX] application serial no. not yet known, filed in US Patent Office 15 Sept.98
[ ] patent no. ______, issued ______

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention. If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invenion is listed below * and no rights to the invention are held by any person, other than the inventor, who could not qualify as small business concern under 37 CFR 1.9 (d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9 (d) or a nonprofit organization under 37 CFR 1.9(e). * NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME
ADDRESS Creatogen Biosciences GmbH, Ulmerstraße 160a, D-86156 Augsburg Federal Republic of Germany
[ ] INDIVIDUAL [x] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

NAME ______
ADDRESS ______
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Dr. Heinrich Kuhn
TITLE IN ORGANIZATION ~~Patentbevollmächtigter der MPG~~
ADDRESS OF PERSON SIGNING ~~Königinstr. 19, 80539 München~~
Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V.

SIGNATURE [signature]
~~Dr. Heinrich Kuhn~~

Export systems for recombinant proteins

Description

The present invention relates to vectors, host-vector combinations and processes for preparing stable fusion proteins consisting of a carrier protein and a passenger protein, where expression of the fusion proteins leads to exposure of the passenger domains on the surface of bacterial cells, especially Escherichia coli cells. If required, the passenger domains can be released into the medium by proteases, for example by selected host factors such as, for example, OmpT.

The present invention further relates to the use of carrier proteins or carrier protein portions from natural proteins which are present as amino-acid sequences in data banks or files and are called, in accordance with their properties, autotransporters.

Methods for identifying and selecting bacteria which express at least one passenger protein on their surface with defined affinity for a binding partner, and the use thereof for diagnostic purposes, are made possible by the present invention. In particular, the process according to the invention allows peptide libraries to be expressed on the surface of bacterial cells, with the aid of which it is possible, for example, to determine the ligands having the highest affinity in the case of antibodies, MHC molecules or other components of the immune system.

Also made possible by the process according to the invention is the production of fusion proteins which are composed of portions of heavy and light antibody domains and an autotransporter, and transport thereof through the bacterial cell coat. In a specific embodiment, finally, the targeted variation of recombinant antibodies with binding activity, and their functional presentation on the cell surface of Escherichia coli become possible.

The process according to the invention generally allows recombinant proteins, which may be

receptors or ligands, to be expressed on the bacterial surface, and selection on the basis of the binding affinity for a binding partner, which makes selection, associated therewith, of a clonal producer possible.

The use of bacteria which express protein fusions on the cell surface and which are present bound to a carrier material or in solution for the specific enrichment or purification of a binding partner showing affinity for protein domains exposed on this surface is also according to the invention. Furthermore, the present invention also relates to the surface expression of enzymes or other proteins with biologically, chemically or industrially relevant properties, and, where needed, the specific release thereof into the surrounding medium.

The exposure of recombinant proteins on the surface of bacterial cells is a method with a large number of possible microbiological, molecular biological, immunological or industrial applications. Production of recombinant proteins in this manner makes their properties, for example binding affinities or enzymatic activities (Francisco et al., Bio. Technology 11 (1993) 491-495) available without a further step such as, for example, disruption of the producer cell being necessary. Since only a limited number of factors are naturally expressed on the bacterial surface, there is in addition specific enrichment of the recombinant protein by comparison with cytosolic production. Another considerable advantage is that the same methods used to select the recombinant protein which is sought can also be used to isolate the producer of this protein, a bacterial cell, and thus a clonal producer which can be permanently stored, stably reproduced and grown on a large scale can be obtained.

Various systems have been used to date for the presentation of recombinant proteins on the cell surface, but these without exception are also used naturally for the transport or secretion of bacterial surface proteins (Little et al., TIBTECH 11 (1993),

3-5). Significantly, in these cases the DNA region which naturally codes for the protein to be transported, the passenger, was replaced or supplemented by the coding DNA region of the required recombinant protein, although the coding regions of the protein domains responsible for the transport, the carrier proteins, usually remained unchanged. It is clear from this that systems in which passenger and carrier components are present immediately adjacent or encoded in one gene, so-called one-component systems, have a considerable advantage by comparison with systems having several independent components (Gentschev et al., Behring Inst. Mitt. 95 (1994) 57-66), especially in the production of universally usable vectors which, besides the property of stable replication, one or more selection markers, and the protein domains needed for transport, must also contain an insertion site for the DNA fragment encoding the passenger. The carrier proteins used in many one-component systems used to date have been E. coli outer membrane proteins. These include, inter alia, LamB (Charbit et al., Gene 70 (1988), 181-189), PhoE (Agterberg et al., Gene 59 (1987), 145-150) or OmpA (Franscisco et al., Proc. Natl. Acad. Sci (1992), 2713-2717), whose use entails disadvantages, however. Thus, additional protein sequences can be integrated only in loops exposed on the surface, which on the one hand leads to fixed amino- and carboxyl-terminal ends on the flanking carrier protein sequences, and on the other hand has a limiting effect on the length of the sequences to be introduced. Although the use of peptidoglycan-associated lipoprotein (PAL) as carrier protein leads to transport to the outer membrane, no presentation of native protein sequences on the surface of E. coli is possible therewith (Fuchs et al., Biol. Technology 9 (1991), 1369-1372). Surface expression of relatively large proteins is possible using a fusion of OmpA and Lpp as carrier protein portion, to whose carboxyl end the passenger protein sequences are

attached (Franscisco et al., Proc. Natl. Acad. Sci (1992), 2713-2717). A disadvantage which has to be accepted in this case is that the fixing of the N-terminus of the passenger may prevent correct folding or functioning.

Also known are so-called autotransporter-containing proteins, a family of secreted proteins in Gram-negative bacteria. The publication of Jose et al. (Mol. Microbiol. 18 (1995), 377-382) mentions some examples of such autotransporter proteins. These proteins contain a protein domain which enables an N-terminally attached protein domain to be transported through a pore structure formed from β-pleated sheet structures in the outer membrane of Gram-negative bacteria. The autotransporter-containing proteins are synthesized as so-called polyprotein precursor molecule. The typical structure of such a precursor protein is divided into three. At the N-terminus there is a signal sequence which is responsible for the transport through the inner membrane, taking advantage of the Sec transport apparatus present in the host and being deleted during this. To this is attached the protein domain to be secreted, followed by a C-terminal helper domain which forms a pore in the outer membrane, through which the N-terminally attached protein domain to be secreted is translocated to the surface. Depending on its function to be carried out, the latter remains there linked to the helper, which is now serving as membrane anchor, on the bacterial surface, or is deleted by proteolytic activity, and this proteolytic activity may be intrinsic to the protein domain to be secreted or be a property derived from the host or be an external/specifically added activity (for example thrombin, IgA protease). Secretion of heterologous polypeptides or proteins using an expression system based on an autotransporter is known. Thus, for example, it is known from EP-A-0 254 090 or the publication of Klauser et al. (EMBO J. 11 (1992), 2327-2335) that the helper domain of the IgA protease

from N. gonorrhoeae can express heterologous polypeptides as passenger domains in the heterologous bacterial strains E. coli and Salmonella typhimurium.

In addition, the extracellular transport of the protein VirG by shigella is described in Suzuki et al. (J. Biol. Chem. 170 (1995) 30874-30880). This protein is likewise an IgA protease-like autotransporter which is capable of the expression of foreign polypeptides such as, for example, MalE and PhoA, which have been covalently linked to the N terminus of the autotransporter domain of VirG. In addition, the paper by Shimada et al. (J. Biochem, 116 (1994), 327-334) describes the extracellular transport of a heterologous polypeptide, namely pseudoazurin from A. faecales, in E. coli using the autotransporter domain of the serine protease from S. marcescens.

In the processes described in the prior art for preparing for the expression of heterologous passenger proteins with the aid of autotransporter systems, however, considerable disadvantages have been found. Thus, on use of the transporter or helper domain of the IgA protease from N. gonorrhoeae in E. coli as host strain, considerable compatibility problems frequently arise. Excessive expression leads to cytolysis or the bacteria show reduced growth even with moderate expression, which in both cases leads to a considerable reduction in the yield of fusion protein and points to weaknesses in the stability of the system. The present invention was thus based on the technical problem of providing carrier proteins which, especially on use of E. coli as host strain, do not lead to these disadvantages because, for a variety of reasons, E. coli is to be preferred to, for example, Neisseria gonorrhoeae as host strain. On the one hand, E. coli strains with recombinant DNA can be cultured even in simple laboratories of safety level 1. In addition, E. coli strains have already been used in the commercial production of recombinant proteins. This means that there is a considerable advantage in the

handling and manipulation of recombinant E. coli strains by comparison with other host strains. In addition, a large number of accurately characterized mutant strains of E. coli already exist and permit a selection of the host strain depending on the required use.

This problem is solved by a method for presenting peptides or/and polypeptides on the surface of Gram-negative host bacteria, where

a) there is provision of a host bacterium which is transformed with a vector on which is located, operatively linked to a promoter, a fused nucleic acid sequence comprising:
   (i) a signal peptide-encoding nucleic acid section,
   (ii) a nucleic acid section coding for the passenger peptide or/and passenger polypeptide to be presented,
   (iii) where appropriate a nucleic acid section coding for a protease recognition site,
   (iv) a nucleic acid section coding for a transmembrane linker and
   (v) a nucleic acid section coding for a transporter domain of an autotransporter; and

(b) the host bacterium is cultivated under conditions with which there is expression of the fused nucleic acid sequence and presentation of the peptide or polypeptide encoded by the nucleic acid section (ii) on the surface of the host bacterium,

characterized in that the nucleic acid section (ii) is heterologous relative to the nucleic acid section coding for the transporter domain (v), and the host bacterium is homologous relative to the nucleic acid section coding for the transporter domain (v).

It is surprisingly possible by using a host bacterium which is homologous relative to the nucleic acid section coding for the transporter domain to achieve a surface presentation of peptides or and

polypeptides, in particular including short synthetic peptides with a length of, preferably, 4-50 amino acids or of eukaryotic polypeptides, which is distinctly improved by comparison with the prior art.

In the process according to the invention there is provision of a host bacterium which is transformed with one or with a plurality of compatible recombinant vectors. A vector of this type contains, operatively linked to a promoter and, where appropriate, other sequences necessary for expression, a fused nucleic acid sequence. This fused nucleic acid sequence comprises (i) a signal peptide-encoding section, preferably a section which codes for a Gram-negative signal peptide which makes passage through the inner membrane into the periplasm possible. The fused nucleic acid sequence (ii) also comprises a section coding for the passenger peptide or polypeptide to be presented. A nucleic acid section coding for a protease recognition site is, where appropriate, located on the 3' side of this section (iii). Examples of suitable protease recognition sites are recognition sites for intrinsic, that is to say naturally present in the host cell, or externally added proteases. Examples of externally added proteases are the IgA protease (compare, for example, EP-A-0 254 090), thrombin or factor X. Examples of intrinsic proteases are OmpT, OmpK or protease X. On the 3' side of this section there is located (iv) a nucleic acid section coding for a transmembrane linker, which makes presentation of the peptide or polypeptide encoded by section (iii) on the outside of the outer membrane of the host bacterium possible. On the 3' side of this section is a nucleic acid section coding for a transporter domain of an autotransporter.

The transmembrane linker domains particularly preferably used are homologous in relation to the autotransporter, that is to say the transmembrane linker domains are encoded by nucleic acid sections directly on the 5' side of the autotransporter domains.

The length of the transmembrane linkers is preferably 30-16 amino acids.

The transporter domain is able to form a so-called β-barrel in the outer membrane of the host bacterium. The β-barrel consists of an even number of antiparallel, amphipatic, β-pleated sheets. This structure has, like other proteins of the outer membrane of Gram-negative bacteria, an aromatic amino acid such as phenylalanine or tryptophan at the C terminus. This is followed alternately by charged (polar) and uncharged (hydrophobic) amino acids, and this structure appears to play a part in the folding with the membrane. The number and location of the amphipatic, β-pleated sheets can be identified with the aid of a suitable computer program and be used, with the aid of analogies to the outer membrane porins whose crystal structure is known (Cowan et al., Nature 358 (1992) 727-733), for constructing a model of the barrel structure. The barrel structure is preferably constructed as follows: 9-14, in particular about 12 amino acids (AA) for a membrane passage; no or a minimal number of charged AA point outwards in a β-pleated sheet; small loops, or none at all, point inwards, where appropriate large or very large loops point outwards; the β-barrel is composed of 12, 14, 16 or 18, in particular 14, antiparallel β-pleated sheets.

Starting from the model of the barrel, it is now possible for the region necessary for self-transport through the outer membrane to be established and linked by a signal peptide and a passenger domain at the genetic level. Expression of this construct then makes transport of the passenger protein to the bacterial surface possible, it being possible for the signal peptide to derive originally from the passenger or from another protein. It must be taken into account in this connection that a linker region which is of suitable length and sequence and which extends through the pore which has formed and ensures that the

passenger domains are completely exposed on the surface is also linked properly to the β-barrel.

An essential feature of the process according to the invention is that the host bacterium is homologous relative to the nucleic acid section coding for the transporter domain, that is to say the host cell and the transporter domain are selected from homologous families, for example enterobacteria, preferably from homologous genera, for example escherichia, salmonella, or helicobacter, particularly preferably from homologous species, for example Escherichia coli, Salmonella typhimurium. It is particularly preferred to use salmonella or E. coli as host bacterium and a transporter domain which is likewise derived from salmonella or E. coli, or a variant thereof.

A particularly suitable E. coli host strain which may be mentioned here is the strain JK321 (DSM 8860) which is $ompT^-$, $dsbA^-$ and carries the genetic marker fpt, which leads to stable surface expression even of large proteins such as, for example, the V_h chain of an antibody with the aid of the igaβ helper protein.

In a preferred embodiment, the present invention therefore relates to a carrier protein which performs an autotransporter function and makes surface exposure of recombinant proteins possible in Escherichia coli with high yield. In a typical example, this is the autotransporter of the "adhesin involved in diffuse adherence" (AIDA-I) from E. coli (Benz and Schmidt, Infect. Immun. 57 (1989), 1506-1511). The transporter domain of the AIDA-I protein is depicted in Fig. 2. Besides this specific sequence, it is also possible to use variants thereof which can be produced, for example, by modifying the amino-acid sequence in the loop structures not involved in the membrane passage. It is also possible, where appropriate, for the nucleic acid sections coding for the surface loops to be completely deleted.

It is also possible within the amphipatic β-pleated sheet structures to carry out conservative amino acid exchanges, that is to say replacement of one hydrophilic by another hydrophilic amino acid or/and replacement of one hydrophobic by another hydrophobic amino acid. A variant preferably has a homology of at least 80% and, in particular, at least 90% with the sequence, indicated in Fig. 2, of the AIDA-I autotransporter domain, at least in the region of the β-pleated sheet structures.

In another typical example, the autotransporter used is that of the SepA protein from Shigella flexneri (Benjellou-Touimi et al., Mol. Micobiol 17 (1995) 123-135) or a variant thereof. In another typical example, it is the autotransporter of the IcsA protein from Shigella flexneri (Goldberg et al., J. Bacteriol 175 (1993), 2189-2196) or of the Tsh protein from E. coli (Provence et al., Infect. Immun 62 (1994), 1369-1380). In another typical example, it is the autotransporter of the Hsr protein from Helicobacter mustelae (O'Toole et al., Mol. Microbiol. 11 (1994), 349-361), of the Prn protein from Bordetella ssp. (Charles et al., Proc. Natl. Acad. Sci USA 86 (1989), 3554-3558; Li et al., J. Gen. Microbiol. 138 (1992), 1697-1705), of the Ssp protein from Serratia marcescens (for example in Yanagida et al., J. Bacteriol. 166 (1986), 937-944 or Genbank Accession No. X59719, D78380), of the Hap protein from Haemophilus influenzae (StGeme et al., Mol. Microbiol. 14 (1994), 217-233), of the BrkA protein from Bordetella pertussis (Fernandez and Weiss, Infect. Immunol. 62 (1994), 4727-4738), of the VacA protein from Helicobacter pylori (Schmitt and Haas, Mol. Microbiol. 12 (1994), 307-319) or various rickettsial proteins (for example 190kDa cell surface antigens, Genbank Accession No. M31227; SpaP, Carl et al., Proc. Natl. Acad. Sci. USA 87 (1990), 8237-8241; rOmpB, Gilmore et al., Mol. Microbiol. 5 (1991), 2361-2370 and Slp T, Hahn et al., Gene 133 (1993), 129-133) or a variant thereof as defined above.

The DNA sequences, and the amino-acid sequences derived therefrom, of the aforementioned auto-transporters are depicted in Figures 7-24.

Further autotransporter domains in bacterial surface proteins or in secreted bacterial proteins may be derived from protein sequences present in data banks from in protein sequences which are based on DNA sequences available in data banks, or from protein sequences determined by sequence analysis directly or indirectly via the DNA sequence. The corresponding coding regions (genes) can be used to prepare vectors or fusion protein genes which make efficient surface expression of passenger proteins possible in Gram-negative bacteria, especially E. coli.

Surface presentation or exposure means according to the invention that the fusion proteins or passenger domains are located on the side of the outer bacterial membrane facing the medium. In intact Gram-negative bacteria, passenger proteins exposed on the surface are freely accessible to binding partners.

In a preferred embodiment, the present invention thus makes possible the surface presentation of peptides or, in another embodiment, the surface presentation of peptide libraries in Gram-negative bacteria, especially in E. coli, and the use thereof for determining the affinity for an antibody or another receptor or for epitope mapping. Epitope mapping means that the peptide with the greatest affinity for an antibody or another receptor is identified exposed on the surface of the producing strain. This makes clear a crucial advantage of the present invention by comparison with previously used phage systems (Makowski, Gene 128, (1953), 5-11) for expressing peptide libraries. In the bacterial system according to the invention, identification of a peptide having the required properties takes place simultaneously with the selection of the clonal producer. The latter can be grown directly and used to produce larger amounts of the required peptide without the need for the elaborate

cycles of infection (phage replication) and selection (phage selection) as with the phage system. The growing of the strain expressing the correct peptide exposed on the surface takes place over the same time as amplification of the corresponding coding gene, sequence analysis of which permits unambiguous identification and characterization of the peptide with simple and established techniques. These advantages according to the invention apply to all passenger domains expressed exposed on the surface using the present invention, that is to say peptides and polypeptides.

A peptide library produced according to the invention thus contains fusion proteins composed of an autotransporter, in a particularly preferred embodiment of the AIDA autotransporter, and of a peptide which is produced, exposed on the surface, in a Gram-negative bacterium, preferably E. coli. The wide variety of different expressed peptides results in a typical example from the cloning of degenerate, synthetically prepared oligonucleotides between the coding regions for the signal peptide and the autotransporter.

In another preferred embodiment, the present invention makes it possible to express proteins or protein fragments acting as antigen on the surface of Gram-negative bacteria, preferably E. coli. The construction of a fusion protein of this type takes place according to the invention using the β subunit of the toxin Vibrio cholerae (CtxB) as passenger and the AIDA autotransporter as carrier protein. The accessibility of the surface-exposed antigenic domains for suitable binding partners has been demonstrated according to the invention by labelling with an antiserum specific for CtxB. It emerged from this that the recombinant fusion proteins embedded in the outer membrane of the E. coli host strain may comprise up to 5% of the total cell protein, which means a considerably improved efficiency by comparison with other systems. The process described here thus makes

possible the stable production and presentation of proteins or protein fragments having antigenic activity on the surface of Gram-negative bacteria and, in a preferred embodiment, the use thereof as live vaccine, for oral vaccination or for screening sera or antibody banks. The use of bacterial cells, for example attenuated salmonella strains (Schorr et al., Vaccine 9 (1991) 675-681) with proteins which have antigenic activity and are expressed exposed on the surface has proved advantageous in live vaccination by comparison with the intracellular bacterial expression of antigens.

The present invention generally permits, in a preferred embodiment, the surface expression of all passengers which are in their essential constituent peptides or proteins on the surface of Gram-negative bacteria, in particular E. coli.

In another preferred embodiment, the C-terminal domain of the AIDA protein, the AIDA autotransporter, serves as membrane anchor for the presentation of recombinant polypeptides of the immune system, for example recombinant antibody domains on the surface of Gram-negative bacteria. Surface expression of recombinant antibody fragments makes it possible to modify them rapidly and to assess and investigate their antigen-binding properties. Thus, it becomes possible to produce whole libraries of functional antibody fragments exposed on the surface, and to test them for particular given binding properties or affinities. The advantage of the present invention by comparison with previously used phage systems is that the variation, that is to say the genetic manipulation and the production of the protein, can take place in the same host organism. It is moreover possible for the genetic manipulation to be targeted (site-specific mutagenesis) or random, using degenerate oligonucleotides to synthesize an intact fusion of antibody-encoding fragment as passenger and the autotransporter as carrier protein. It is likewise possible for the

genetic manipulation to take place in the form of in vivo mutagenesis by exposing the bacteria which contain the gene for the fusion protein to high-energy radiation (for example UV) or chemical agents having mutagenic effects.

The selection, according to the invention, of the molecule having the correct binding properties takes place alongside the selection of the producing bacterial cell. It is evident from this that this procedure according to the invention, in its strategy consisting of variation and subsequent selection, is based on the natural strategy of the immune system for the best possible adaptation of binding properties of immunogenic molecules. Various procedures according to the invention are conceivable for expressing functional antigen-binding parts of antibodies, which are not usually glycosylated, on the surface of Gram-negative bacteria, preferably E. coli. Two monovalent fragments can be presented together through separate fusions of the light chain (VL) and the heavy chain (VH) with, in each case, an autotransporter domain, which are expressed independently of one another with different compatible vectors or under the control of different promoters on the same vector in a host cell. The two antibody domains which are present exposed on the surface assemble to form a functional Fv fragment on the surface, it being possible for the stability of the interaction to be promoted by chemically induced disulphite bridge formation or another type of chemical crosslinking.

In another procedure according to the invention there is preparation of fusion proteins which contain the autotransporter as carrier protein, and as passenger the light chain (VL) and the heavy chain (VH) of an antigen-binding domain of an antibody, linked via a short linker peptide (for example $[Gly_4Ser]_3$) which permits correct assembly of the two chains to form a functional Fv fragment. For construction of such single-chain (sc) Fv fragments, it is possible both to

link the N terminus of the light chain to the C terminus of the heavy chain, and to link the N terminus of the heavy chain to the C terminus of the light chain (Pluckthun Immun. Rev. 130 (1992), 151-188). It is also possible using the procedures described to produce a complete Fab fragment.

In another preferred embodiment, the present invention makes possible the surface-exposed expression of MHC class II molecules in E. coli, where appropriate with defined embedded peptides. Two strategies are conceivable for this. In one variant, two different fusion proteins, both of which contain an autotransporter as carrier protein, are expressed on separate compatible vectors or on one vector under the control of different promoters in a host cell. The passenger protein employed is, on the one hand, the ∀ chain of the required MHC class II subtype and, on the other hand, the β chain of this subtype, to whose N terminus the required peptide can be attached via a linker (Kozono et al., Nature 369 (1994) 151-154).

In the second variant, a passenger protein consisting of the peptide, the β chain and the ∀ chain is fused to an autotransporter. The ∀ chain and β chain assemble on the bacterial surface to form a functional MHC molecule, with the peptide being correctly embedded in the binding cavity. The stability of the complex can be assisted by a chemically induced disulphide bridge formation. Variation of the embedded peptide is possible by site-specific mutagenesis or/and by using degenerate oligonucleotide primers in the preparation of the DNA fragments encoding the fusion proteins, as well as by in vivo mutagenesis methods using high-energy radiation or/and chemical mutagens.

Once again, the advantage of the process according to the invention becomes clear. Variation of the binding partner, expression, selection of the molecule having the optimal properties, sequence analysis and stable production can take place in one host strain. This also makes it possible, for example

for variants of previously known ligands with improved binding properties to be rapidly characterized, and thus optimization of ligands or receptors.

In another preferred embodiment, the present invention makes possible the surface expression of immunomodulatory receptors such as, for example, CD1, Fc receptor or MHC class I molecules, and specific variation thereof.

In another preferred embodiment, the present invention makes possible the surface expression of T-cell receptors or parts thereof, but also of other surface antigens of eukaryotic cells or cells of the immune system.

In another preferred embodiment, the protein fragments or peptides expressed on the surface are T-cell epitopes which, following uptake of the bacteria by appropriate cell lines or primary cells such as, for example, macrophages, presented as peptides embedded in MHC molecules of class I or II and can serve to stimulate specific T cells.

In a particularly preferred embodiment, the process according to the invention makes possible the surface expression and the variation of a peptide or polypeptide having an affinity for a binding partner, of a ligand, of a receptor, of an antigen, of a toxin-binding protein, of a protein having enzymatic activity, of a nucleic acid-binding protein, of an inhibitor, of a protein having chelator properties, of an antibody or of an antigen-binding domain of an antibody.

The term "binding partner" means according to the invention an element, a molecule, a chemical compound or a macromolecule, where the binding partner and/or the bacterial cells expressing the fusion proteins are in a freely soluble form, bound to a matrix or else associated with a biological membrane.

The term "antigen-binding domain" refers according to the invention to at least the region of an

antibody molecule which is sufficient for specific binding of an antigen.

In another preferred embodiment, the present invention makes chemical, physical or/and enzymatic modification of the passenger peptide or polypeptide, or parts thereof, exposed on the surface possible, it being possible for the modification to be a covalent modification, a non-covalent modification, a glycosilation, a phosphorylation or a proteolysis.

The process according to the invention for producing a variant population of peptides exposed on the surface and for identifying bacteria which carry peptides or polypeptides having a particular required property is divided into the following steps:

(1) preparation of one or more fusion genes by cloning the coding sequence of a required passenger in frame with the coding sequence of a transporter domain of an autotransporter and of a signal peptide, it being possible for the individual subfragments to be amplified by PCR or to derive from restriction digestions of other DNA, in at least one vector;

(2) variation of the passenger by mutagenesis, for example by site-specific mutagenesis, using degenerate oligonucleotide primers in the PCR, by chemical mutagenesis or by using high-energy radiation;

(3) introduction of the vector or vectors into host bacteria;

(4) expression of the fusion gene or fusion genes in the host bacteria which present the fusion protein or fusion proteins stably on the surface;

(5) cultivation of the bacteria, for example in liquid culture or on agar plates, to produce the

passenger presented stably exposed on the surface or the passengers presented stably exposed on the surface;

(6) where appropriate selection of the bacteria which carry the passenger or passengers having the required properties on the surface, and

(7) where appropriate characterization of a binding partner for the passenger having the optimal properties.

It is moreover possible according to the invention to perform this process several times in order to adapt the properties of the surface-exposed peptide or polypeptide stepwise to the required binding behaviour, or to optimize, in a first step, the binding partner in respect of one property and, in a second step, in respect of one or more other properties. However, it is also possible according to the invention, depending on the required use, to link only a few constituent steps of the process together, in a typical example the constituent steps (1), (3), (4) and (5), but also all other possible combinations.

In a preferred embodiment of this process, the fusion protein contains as carrier protein the autotransporter domain of the AIDA protein or a variant thereof which makes secretion of the fusion protein possible.

In another preferred embodiment of this process, the fusion protein contains as carrier protein the SepA autotransporter or a part thereof, or the IcsA autotransporter or a part thereof, or the Tsh autotransporter or a part thereof, or the Ssp autotransporter or a part thereof, or the Hap autotransporter or a part thereof, or the Prn autotransporter or a part thereof, or the Hsr autotransporter of a part thereof, or the BrkA autotransporter or a part thereof, or the VacA

autotransporter or a part thereof or a rickettsial autotransporter or a part thereof, each of which makes secretion of the fusion protein possible.

The expression of multimeric proteins is possible according to the invention by preparing in one cell different fusion proteins which assemble on the surface to form a functional unit.

The short generation time of the bacteria used as host organism makes it possible to have a permanent variation and selection cycle which makes it possible to adapt, in an evolutionary manner, the passenger potein, but also the autotransporter, to given properties. This may involve, in a typical example, the binding affinities between the passenger protein and a binding partner. The isolation of the bacteria having the stably exposed fusion protein takes place, in a preferred embodiment of this process, by binding to an immobilized or/and labelled binding partner, for example a matrix-fixed binding partner, to a binding partner with a fluorescent label, a binding partner labelled with magnetic particles, or a binding partner with a chromogenic label. In another preferred embodiment, the binding partner is modified so that it can be detected in a second step by a binding partner specific for the modification.

Another aim of the present invention is to provide stably expressed fusion proteins or parts thereof or fusion proteins expressed stably on the surface of bacteria, and the use thereof for therapeutic purposes or diagnostic purposes, in pollutant concentration or removal, in the inactivation of toxins, in the mobilization of raw materials, in food production or processing, in detergent production, in the labelling of selected eukaryotic or prokaryotic cells. It is possible according to the invention to use bacteria expressing antibodies or antibody fragments stably on the surface, in a typical example using the AIDA autotransporter as transporter domain, for the production thereof, these antibodies or antibody

fragments subsequently being employed, where appropriate after purification, for diagnostic or therapeutic purposes. It would be possible, for example, to use such antibodies or antibody fragments to identify or select specifically cells with particular surface markers, a typical example which may be mentioned here being tumour antigens. In another typical example, the labelled surface markers are receptors, in which case the labelling takes place along with the blocking of the or one of the receptor properties, which makes it possible specifically to inhibit a signal transduction induced or mediated by the receptor, and the cell function associated therewith.

Description of the figures

Figure 1:
Hydrophobicity of the C-terminal 300 amino acids of the AIDA-I protein.

The pore typical of autotransporters in the outer membrane of Gram-negative bacteria is formed by amphipatic β-pleated sheet structures, that is to say by domains with β-pleated sheet structure and alternating hydrophobic and hydrophilic amino acids. This can be demonstrated by plotting a relative hydrophobicity value of the amino acid, which has been assigned to the amino acid by means of a particular algorithm, against the position of the amino acid. The algorithm of Vogel and Jähnig (J. MoL Biol. 190 (1986) 191-199) was used. The arrows show the possible membrane passages, with an arrow to the left denoting that the membrane passage runs from the inside to the outside and an arrow to the right indicating a membrane passage from the outside to the inside. SP indicates a relative surface probability of the amino acids calculated by the method of Emini et al. (J. Virol. 55 (1985), 836-839).

Figure 2:
Model of the autotransporter from the AIDA-I protein.

Starting from the plot of the relative hydrophobicity of an amino acid against its position (Figure 1), the barrel structure formed by the antiparallel, amphipatic β-pleated sheets can be depicted as model. The barrel structure which is depicted here cut open is closed in the membrane by interaction of the first with the antiparallel last membrane passage. The amino acids written inside rhombi are located in the membrane region, with those surrounded by thick lines being relatively hydrophobic and being oriented towards the outside of the barrel, that is to say towards the membrane, while those surrounded by thin lines are relatively hydrophilic and point with their side chains towards the inside of the pore. Amino acids shown in circles form loops outside the membrane. Alanine at position 1 of the model has the number 1014 in the complete sequence of the AIDA-I, while the terminal phenylalanine has the number 1286 in the complete sequence (Benz and Schmidt, Mol Microbiol 11 (1992), 1539-1546).

Figure 3a:
Preparation of pJM7, a vector for surface expression of CtxB.

pJM7 contains a gene fusion (FP59) of cholera toxin B and the AIDA linker/β-barrel region. This gene fusion is expressed constitutively under the control of the artificial promoter PTK (Klauser et al., EMBO J. 9 (1990) 1991-1999) in a vector with high copy number. The ctxB gene was amplified by PCR using the oligonucleotides EF16 and JM6 from the plasmid pTK1 (Klauser et al. EMBO J. 9 (1990) 1991-1999). The autotransporter consisting of the β-barrel and the linker region from AIDA-I was amplified by amplification using the oligonucleotides JM1 and JM7 from a plasmid DNA preparation from E. coli EPEC 2787 (Benz and Schmidt, Infect. Immun. 57 (1989),

1506-1511). The oligonucleotide JM1 contains in its 5' projection a BglII recognition sequence, and oligonucleotides JM6 and JM7 each contain a KpnI recognition sequence. The vector DNA (pBA) was hydrolysed with ClaI and BamHI, and the two PCR products were then, following the amplification, cut with ClaI and KpnI (EF16/JM6 fragment) or with BglII and KpnI (JM7/JM1 fragment). The three fragments generated in this way were condensed in a ligation.

Figure 3b:
Preparation of pJM22, a vector for surface expression of peptides.

pJM22 produces the fusion protein FP50 which consists of three domains. At the N-terminal end there is located the CtxB signal sequence which ensures export of the resulting fusion protein through the cell membrane (Sec mediator). This is followed by the passenger domain, in this case a peptide, the epitope PEYFK. At the C-terminal end of the fusion protein is the AIDA β-barrel/linker region, the autotransporter, which conveys the passenger domain with N-terminal truncation by the signal peptide to the surface of E. coli. To construct pJM22, firstly the DNA of pJM7 was hydrolysed with XhoI, and the vector portion of pJM7 was amplified by PCR using the oligonucleotides JM7 and JM20. This entailed deletion of the ctxB gene apart from its signal sequence. The oligonucleotide JM20 contained in its 5' overhang, in addition to the KpnI cleavage sequence, five codons which code for the amino acids PEYFK. This amino-acid sequence represents a linear epitope for the monoclonal antibody Dü142. The PCR product was hydrolysed with KpnI and then self-ligated.

Figure 4
Expression detection and protease sensitivity

Because of the strong stable expression of the fusion proteins FP59 (derived from pJM7) and FP50

(derived from pJM22) in E. coli, these can easily be identified in a whole cell lysate stained with Coomassie brilliant blue. Protease accessibility represents a conventional means for determining the location of a protein. Access is to be expected to cell-intrinsic proteins only if these are presented on the outside of the bacterium or if the outer membrane of the bacterium is permeable to proteases. To rule out the latter, it is possible to use a protease-sensitive marker which is known to be naturally present in the periplasm. The integrity of the outer membrane is ensured only if this marker is not attacked by the protease employed. Cells of E. coli UT5600 or JK321 were cultured overnight on LB agar (50 mg/l ampicillin) and suspended in PBS. The cell suspensions were adjusted to an OD578 = 4.0. Cells from 0.5 ml of cell suspension were sedimented for 1 min in a bench centrifuge and resuspended in 200 µl of PBS with 0.1 mg/ml protease. The mixtures were incubated at 37°C for 20 min and stopped by cooling to 0°C, sedimenting for 1 minute and resuspending the pellet in 40 µl of SDS-PAGE sample buffer and immediately boiling for 15 minutes. The evaluation took place after SDS-PAGE by Western blotting (4b and 4c) or by staining with Coomassie brilliant blue (4a). Access of the proteases to the periplasm was ruled out by employing not only antisera specific for the passenger protein domains but also an antiserum specific for the C-terminal part of OmpA, which is naturally present inaccesibly in the periplasm and ought therefore not be capable of being attacked by externally added proteases such as trypsin (4c).

Figure 4a:

SDS-PAGE and subsequent staining with Coomassie brilliant blue to detect protease sensitivity and quantify expression. Whole cell lysates of E. coli JK321 and E. coli UT5600 were loaded.

Lane 1 JK321 pJM7 C *
Lane 2 JK321 pJM7 T**
Lane 3 JK321 pJM7 -***
Lane 4 Molecular weight markers (94, 67, 43, 30, 20 and 14 kDa)
Lane 5 JK321 pJM22 C
Lane 6 JK321 pJM22 T
Lane 7 JK321 pJM22 -
Lane 8 JK321 pTK61 C
Lane 9 JK321 pTK61 T
Lane 10 JK321 pTK61 -
Lane 11 UT5600 pJM7 C
Lane 12 UT5600 pJM7 T
Lane 13 UT5600 pJM7 -
Lane 14 Molecular weight markers (94, 67, 43, 30, 20 and 14 kDa)
Lane 15 UT5600 pJM22 C
Lane 16 UT5600 pJM22 T
Lane 17 UT5600 pJM22 -
Lane 18 UT5600 pTK61 C
Lane 19 UT5600 pTK61 T
Lane 20 UT5600 pTK61 -

C* Cells were digested with chymotrypsin
T** Cells were digested with trypsin
*** Native cells

Figure 4b:
Western blot for detecting expression and protease sensitivity

Whole cell lysates of E. coli JK321 and E. coli UT5600 were loaded. After the electrophoresis, the proteins were transferred from the gel by the semi-dry method to a nitrocellulose membrane. The filters were then blocked with blocking solution (PBS with 0.5% Tween 20 and 0.5 M NaCl) for 10 min, and the first antiserum, AK55 (rabbit anti-cholera toxin B) diluted 1:200 in blocking solution, was added. To detect the epitope PEYFK, the hybridoma supernatant Dü142, diluted

1:35 in blocking solution, was added. The filters were incubated with the primary antibodies for 1 h, then washed three times and incubated with protein A-alkaline phosphatase conjugate (1:500 in blocking solution) for 30 min. The filters were developed with NBT/BCIP colour solution.

Lane 1 JK321 pJM7 C *
Lane 2 JK321 pJM7 T**
Lane 3 JK321 pJM7 -***
Lane 4 Molecular weight markers (106, 80, 50, 32, 27 and 18 kDa)
Lane 5 JK321 pJM22 C
Lane 6 JK321 pJM22 T
Lane 7 JK321 pJM22 -
Lane 8 JK321 pTK61 C
Lane 9 JK321 pTK61 T
Lane 10 JK321 pTK61 -
Lane 11 UT5600 pJM7 C
Lane 12 UT5600 pJM7 T
Lane 13 UT5600 pJM7 -
Lane 14 Molecular weight markers (106, 80, 50, 32, 27 and 18 kDa)
Lane 15 UT5600 pJM22 C
Lane 16 UT5600 pJM22 T
Lane 17 UT5600 pJM22 -
Lane 18 UT5600 pTK61 C
Lane 19 UT5600 pTK61 T
Lane 20 UT5600 pTK61 -

C* Cells were digested with chymotrypsin
T** Cells were digested with trypsin
-*** Native cells

Figure 4 c:
Demonstration of the integrity of the outer membrane by Western blot analysis.

Whole cell lysates of E. coli JK321 and E. coli UT5600 were loaded. After the electrophoresis, the

proteins were transferred from the gel by the semi-dry method to a nitrocellulose membrane. The filters were then blocked with blocking solution (PBS with 0.5% Tween 20 and 0.5 M NaCl) for 10 min, and the first antiserum, K56 (rabbit anti-OmpA) diluted 1:1000 in blocking solution, was added. The filters were incubated with the primary antibodies for 1 h, then washed three times and incubated with protein A-alkaline phosphatase conjugate (1:500 in blocking solution) for 30 min. The filters were developed with NBT/BCIP colour solution. OmpA is an outer membrane protein of E. coli with a C-terminal periplasmic portion. This periplasmic part is trypsin-sensitive. If trypsin has access to the periplasm, a part about 10-11 kDa in size is digested off mature OmpA (35 kDa). Digestion would thus result in a displacement of the OmpA band in the Western blot from 35 kDa to 25 kDa (Klauser et al., EMBO J. 9 (1990) 1991-1999), which is obviously not the case on use of the AIDA-I autotransporter for transporting recombinant proteins.

Lane 1 JK321 pTK1 T*
Lane 2 JK321 pJM7 T
Lane 3 JK321 pJM22 T
Lane 4 JK321 pTK61 T
Lane 5 Molecular weight markers (106, 80, 50, 32, 27 and 18 kDa)
Lane 6 JK321 pTK1 -**
Lane 7 JK321 pJM7 -
Lane 8 JK321 pJM22 -
Lane 9 JK321 pTK61 -
Lane 10 empty
Lane 11 UT5600 pTK1 T*
Lane 12 UT5600 pJM7 T
Lane 13 UT5600 pJM22 T
Lane 14 UT5600 pTK61 T
Lane 15 Molecular weight markers (106, 80, 50, 32, 27 and 18 kDa)
Lane 16 UT5600 pTK1 -**

Lane 17 UT5600 pJM7 -
Lane 18 UT5600 pJM22 -
Lane 19 UT5600 pTK61 -

T* Cells were digested with trypsin
** Native cells

Figure 5
Immunofluorescence

Immunofluorescence of whole, non-permeabilized cells represents a conventional method for detecting determinants exposed on the cell surface. Antibodies employed therein for detecting the determinants are too large to pass through the intact outer membrane. The control used for differentiation and for estimation of the background activity of periplasmically or cellularly expressed determinants comprises antibodies against antigens known to be expressed periplasmically or cellularly respectively.

Cells of E. coli UT5600 which contain one of the plasmids pBA, pTK1, pTK61, pJM7 or pJM22 were cultured overnight on LB agar (ampicillin 50 mg/l) and suspended in PBS to an optical density of 0.1 at 578 nm. 500 µl of this cell suspension were used to coat cover glasses which were placed in 24-well microtitre plates. The cells were sedimented onto the cover glasses in a plate centrifuge for 5 min. 450 µl of the supernatant were aspirated off and replaced by PBS with 2.5% PFA (paraformaldehyde), with which fixation was carried out for 20 min. The supernatant was completely aspirated off and three washes with 500 µl of PBS were carried out. Nonspecific binding sites were blocked by incubation with 300 µl of PBS containing 1% FCS for 5 min. The blocking solution was completely aspirated off, and the cover glasses were centred in their wells, covered with 15 µl of a 1:100 dilution of the rabbit serum AK55 (raised against cholera toxin B) and incubated in a humidity chamber at room temperature for 1 h. This was followed by three

washes with 500 µl of PBS each time, blocking with 350 µl of PBS/FCS for 5 min, and incubation with 15 µl of 1:100 dilution of a goat anti-rabbit-Texas red conjugate for 30 min. After a subsequent three washes, the cover glasses were placed on slides and embedded using embedding medium. The result of the immunofluorescence was assessed under the microscope and recorded by photography with exposure times of equal length.

a) E. coli UT5600 pBA (strain used as negative control containing only the cloning vector without insert)

b) E. coli UT5600 pTK1 (produces cholera toxin B which is exported into the periplasm. This construct is used for determining the background activity of the periplasmically expressed cholera toxin B).

c) E. coli UT5600 pJM7 (expresses FP59, the fusion protein of AIDA and cholera toxin B, which is presented on the surface of E. coli).

d) E. coli UT5600 pJM22 (expresses FP50, the fusion protein of AIDA and the epitope PEYFK. This construct is used to demonstrate that the AIDA portion of FP59 and FP50 shows no cross-reactivity with the AK55 used in this experiment).

e) E. coli UT5600 pTK61 (produces a fusion protein of cholera toxin B and Iga-β which is presented on the surface of E. coli (Klauser et al., EMBO J. 9 (1990) 1991-1999). Used for comparison with the AIDA construct FP59).

Figure 6
DNA sequences of the oligonucleotides used

Figures 7-24
DNA sequence (non-coding strand) and amino-acid sequences derived therefrom, of bacterial autotransporters.

Figure 7
Depiction of the membrane-integrated part of the AIDA-I autotransporter from Escherichia coli (Benz and Schmidt, Mol. Microbiol. 6 (1992), 1539-1546).

Figure 8
Depiction of the membrane-integrated part of the BrkA autotransporter from Bordetella pertussis (Fernandez and Weiss, Infect Immun. 62 (1994), 4727-4738).

Figure 9
Depiction of the membrane-integrated part of the Hap autotransporter from Haemophilus influenzae (StGeme et al., Mol. Microbiol. 14 (1994), 217-233).

Figure 10
Depiction of the membrane-integrated part of the Hsr autotransporter from Helicobacter mustelae (O'Toole et al., Mol. Microbiol 11 (1994), 349-361).

Figure 11
Depiction of the membrane-integrated part of the IcsA autotransporter from Shigella flexneri (Goldberg et al., J. Bacteriol 175 (1993), 2189-2196).

Figure 12
Depiction of the membrane-integrated part of the Prn (outer membrane protein P96) autotransporter from Bordetella pertussis (Charles et al., Proc. Natl. Acad. Sci. USA 86 (1989), 3554-3558).

Figure 13
Depiction of the membrane-integrated part of the Prn (P70 pertactin) autotransporter from Bordetella parapertussis (Li et al., J. Gen. Microbiol. 138 (1992), 1697-1705).

Figure 14
Depiction of the membrane-integrated part of the 190 kDA cell surface antigen autotransporter from Rickettsia rickettsii (Anderson et al., unpublished, Genbank Accession No. M31227).

Figure 15
Depiction of the membrane-integrated part of the SpaP autotransporter from Rickettsia prowazekii (Carl et al., Proc. Natl. Acad. Sci. USA 87 (1990), 8237-8241).

Figure 16
Depiction of the membrane-integrated part of the 120 kilodalton outer membrane protein (rOmp B) autotransporter from Rickettsia rickettsii (Gilmore et al., Mol. Microbiol. 5 (1991), 2361-2370).

Figure 17
Depiction of the membrane-integrated part of the SlpT autotransporter from Rickettsia typhi (Hahn et al., Gene 133 (1993), 129-133).

Figure 18
Depiction of the membrane-integrated part of the SepA autotransporter from Shigella flexneri (Benjellou-Touimi et al., Mol. Microbiol 17 (1995), 123-135).

Figure 19
Depiction of the membrane-integrated part of the Ssp autotransporter from Serratia marcescens RH1 (Rho, unpublished, Genbank Accession No. X59719).

Figure 20
Depiction of the membrane-integrated part of the Ssp autotransporter from S. marcescens IFO-3046, clone pSP11 (Yanagida et al., J. Bacteriol. 166 (1986), 937-944).

Figure 21
Depiction of the membrane-integrated part of the Ssp-h1 autotransporter from Serratia marcescens, strain IFO3046 (Onishi and Horinouchi, unpublished, Genbank Accession No. D78380).

Figure 22
Depiction of the membrane-integrated part of the Ssp-h2 autotransporter from Serratia marcescens, strain IFO3046 (Onishi and Horinouchi, unpublished, Genbank Accession No. D78380).

Figure 23
Depiction of the membrane-integrated part of the Tsh autotransporter from Escherichia coli (Provence et al. 1994, Infect. Immun. 62 (1994), 1369-1380).

Figure 24
Depiction of the membrane-integrated part of the VacA autotransporter from Helicobacter pylori (Schmitt and Haas, Mol. Microbiol. 12 (1994), 307-319). At least 3 other forms of VacA are also known in Helicobacter pylori, but they differ in the stated region to an inconsiderable extent.

EXAMPLES

Example 1:
Identification and localization of the autotransporter in a surface protein of Escherichia coli.

In order to find an autotransporter appropriate for the required use, that is to say adapted to the passenger protein and the host strain to be used, it is necessary to carry out an analysis of the C-terminal amino-acid sequence of a protein under consideration. This may be a protein already identified as surface factor, or else an amino-acid sequence, deposited in a data bank, of a protein of unknown function, or an amino-acid sequence, derived from a DNA sequence

deposited in a data bank, of a protein, or the amino-acid sequence, derived from a gene following a sequence analysis, of a protein. The N terminus of the protein ought to contain a signal peptide sequence in order to make transport across the inner membrane possible, and the part integrated into the membrane ought to start at the C terminus with the aromatic amino acid phenylalanine or tryptophan, followed by alternately polar (or charged) and hydrophobic (or aromatic) amino acids. The passenger domain ought to contain few cysteines and no disulphide bridges at all, since it has emerged that this blocks transport of the passenger through the pore which is formed. The hydrophobicity plot ought to indicate an even number of amphipatic β-pleated sheet structures from which the outer membrane pore is constituted. The amphipatic β-pleated sheet structures ought to be about about 12 amino acids long and contain a minimum amount of charged amino acids oriented towards the membrane side, with the loops joining the membrane passages containing few amino acids towards the periplasm. Considerably more amino acids can be present towards the outside (medium). The results of this in the hydrophobicity plot are an assembly of the membrane passages in antiparallel pairs with the exception of the first and the last membrane passage, which complete the barrel structure of the pore by assembling together in antiparallel fashion. Based on compliance with these criteria, it is now possible to construct a model of the autotransporter, which can be used to establish the location and extent of the amino acids necessary for the transport. In addition to the amino acids needed for the pore, the fusion protein must also include, for an autotransporter capable of functioning, a so-called linker region which runs from the N terminus, located in the periplasm, of the β-barrel structure through the pore to the surface, so that the surface exposure of all the passenger domains is completely ensured.

The first aim of the present invention was to provide a system for optimized surface exposure of recombinant proteins in E.coli. This is why an autotransporter was sought in a natural surface protein of E.coli. The choice fell on the adhesin AIDA-I (Adhesin Involved in Diffuse Adherence, Benz and Schmidt Infect. Immun. 57 (1989) 1506-1511), whose sequence was available in data banks. A signal sequence of 49 amino acids at the N terminus was shown according to the invention, while the requirements according to the invention at the C terminus were met by the amino-acid sequence FSYKI (phenylalanine-serine-tyrosin-lysine-isoleucine).. The transported domain contained no cysteines, and the hydrophobicity plot (Figure 1) predicted 14 antiparallel, amphipatic β-pleated sheet structures. Thus, to form the pore, at least the amino acids from alanine at position 1014 of the complete amino-acid sequence (Benz and Schmidt, Mol. Microbiol. 6 (1992) 1539-1546) up to phenylalanine at position 1286 are necessary (Figure 2). Additionally selected as linker region were amino acids attached to alanine 1014 on the N-terminal side. The functional autotransporter region selected in this way could then be isolated by PCR from the DNA of the corresponding E.coli EPEC2787 and used to construct a fusion protein.

Example 2:

Construction of a surface-exposed fusion protein having an antigenic determinant as passenger protein

Based on the assumptions that AIDA-I is an autotransporter and that a gene fusion of any desired passenger and an autotransporter intrinsic to E.coli (namely AIDA-β) ought to be more compatible with E.coli than a gene fusion of the same passenger with a heterologous autotransporter (for example Iga-β), a gene fusion was produced between aida-β and a gene for a passenger protein. In order to ensure transport of the passenger, not only AIDA-β but also a connecting

region ("linker") located on the N-terminal side of the β-barrel was cloned.

CtxB was selected as passenger, and the corresponding gene from pTK1 (Klauser et al, EMBO J. 9 (1990), 1991-1999) was amplified by PCR using the oligonucleotides EF16 and JM6. Since AIDA-I is plasmid-encoded in E.coli EPEC 2787 (Benz and Schmidt, Infect. Immun. 57 (1989), 1506-1511), the AIDA-I autotransporter with linker region was likewise amplified from a plasmid preparation of E.coli EPEC 2787 by PCR using the oligonucleotides JM1 and JM7. The two PCR products were digested with restriction enzymes whose recognition sequences were present in the oligonucleotides. The two fragments produced in this way were cloned into an appropriately predigested cloning vector (pBA) with high copy number. This resulted in a construct with an artificial constitutive promoter (PTK; Klauser et al., EMBO J. 9 (1990) 1991-1999) in front of a gene fusion consisting of ctxB at the 5' end (coding for amino acids 1-113), followed by an AIDA-I linker (coding for amino acids 116-279 of the fusion protein) and the AIDA-I autotransporter (coding for amino acids 280-563 of the fusion protein) at the 3' end (Figure 3a). The resulting gene fusion was called FP59.

The expression, which was substantially greater than with the previously existing Iga-β system and which was achieved without the tendency to lysis which is to be observed with Iga-β, was unambiguously demonstrated by comparative electrophoresis of whole cell lysates (Figure 4a).

Demonstration of the surface exposure of FP59 was provided by various methods. The protease sensitivity of FP59 was shown in the protein gel by a reduction in the molecular weight following addition of trypsin or chymotrypsin (Figure 3a). Protease-resistant fragments with, in each case, a mass of about 33-35 kDa were produced (Figure 3a). These protease-resistant fragments contain no immunogenic portions of the

passenger protein. This was shown by Western blot analysis of whole cell lysates using an anti-cholera toxin B serum and comparing with protease-digested and undigested FP59-expressing E.coli (Figure 4b and comparison of 4a and 4b).

Partial N-terminal sequencing of the membrane-protected trypsin-digested products revealed that the membrane linker region in the AIDA autotransporter has a length of 55 amino acids.

It was also possible with the protease digestions to show the integrity of the outer membrane of FP59-expressing E.coli (Fig. 4c). For this purpose, whole cell lysates were, following the trypsin digestion, developed by immunoblotting with an anti-OmpA serum. Both undigested cells and trypsin-digested cells showed intact OmpA as was to be expected for cells with an intact outer membrane.

It was also possible to show surface exposure and strong expression of FP59 by immunofluorescence studies (Figure 5). It is possible by binding fluorescence-labelled antibodies to demonstrate the surface exposure of an antigen on a bacterial cell with an intact outer membrane. This was shown by FP59-expressing E.coli cells by strong fluorescence. The E.coli cells used as negative controls, with periplasmically expressed cholera toxin B, with surface-exposed FP50 (Figure 3b) and with non-recombinant cloning vector were unambiguously negative. The periplasmic cholera toxin B demonstrated the inaccessibility of the periplasm for antibodies (Figure 5b), and the negative result of the immunofluorescence with FP50 made it possible to rule out cross-reactivity of the antiserum used (against the passenger protein) with the AIDA portions of FP59 (Figure 5d). The immunofluorescence with the non-recombinant cloning vector was a measure of the background staining intrinsic to the method of measurement (Figure 5a). This also made it possible to compare the expression of FP59 with B61, the surface-

presented cholera toxin B-Iga-β fusion protein produced by pTK61 (Figures 5c and 5e), likewise making it possible to demonstrate an unambiguous advantage of the novel system according to the invention.

Example 3:

Construction of a surface-presented peptide fusion

A peptide which acts as linear epitope for a monoclonal antibody (Dü142) was presented and detected on the surface. The peptide was cloned using a PCR-dependent strategy which is extremely suitable for the generation and surface exposure of peptide libraries. This entails formation of a triple gene fusion of the export signal of ctxB (bases 1-81), of a short sequence coding for a peptide (bases 82-96) and of the aida linker/aida-β region (bases 103-1450).

pJM7 (Figure 3a) was linearized with XhoI and used as template (Figure 3b) for a PCR with the oligonucleotides JM7 and JM20 (Figure 6). Both oligonucleotides had a KpnI recognition sequence at their 5' ends [lacuna] JM7 was chosen so that, on use thereof in a PCR, the aida linker/aida-β domains were amplified. JM20 was chosen so that the PCR product contained the signal sequence present in ctxB for the Sec-dependent membrane transport through the cytoplasmic membrane and the six codons subsequent thereto. In addition, JM20 contained in its 5' extension, which was not complementary to the template, five codons which coded for the linear epitope of the antibody Dü142. The KpnI recognition sequence was located upstream of these codons. After the PCR, the resulting product was hydrolysed with KpnI, self-ligated and then transformed into E.coli. Correct gene fusions were identified by colony immunoblotting (no figure). Expression and surface exposure were demonstrated in analogy to the methods described in Example 2 by Western blot analysis of protease digests

and analysis of protein stainings in the gel (Figures 4a, b, c).

The generation of extensive peptide libraries can be done by slightly modifying the cloning strategy described herein. The division described for JM20 of the various functional regions of this oligonucleotide must for this purpose be altered so that the region coding for the linear epitope is replaced by a region which is deliberately subjected to degeneration during the oligonucleotide synthesis. Degeneration means that, in place of defined bases at all position of this functional region, there is replacement of single, multiple or all bases by a base mixture composed of up to four different bases. This means that each codon can code for up to 20 different amino acids, instead of for one amino acid, resulting in a pool of coding sequences which are theoretically possible for all conceivable combinations of amino acids in a peptide of the given length. The cell which carries the peptide having the required property can now be isolated, mediated by binding of the surface-exposed peptide to a binding partner, which, for example, is in a form immobilized on a matrix, has a fluorescent label or is coupled to magnetic beads, and be used for continual production and characterization.

Patent claims

1. Process for the presentation of peptides or/and polypeptides on the surface of Gram-negative host bacteria, where

a) there is provision of a host bacterium which [lacuna] transformed with a vector on which is located, operatively linked to a promoter, a fused nucleic acid sequence comprising:

(i) a signal peptide-encoding nucleic acid section,

(ii) a nucleic acid section coding for the passenger peptide or/and passenger polypeptide to be presented,

(iii) where appropriate a nucleic acid section coding for a protease recognition site,

(iv) a nucleic acid section coding for a transmembrane linker and

(v) a nucleic acid section coding for a transporter domain of an autotransporter; and

(b) the host bacterium is cultivated under conditions with which there is expression of the fused nucleic acid sequence and presentation of the peptide or polypeptide encoded by the nucleic acid section (ii) on the surface of the host bacterium,

characterized in that the nucleic acid section (ii) is heterologous in relation to the nucleic acid section coding for the transporter domain (v), and the host bacterium is homologous in relation to the nucleic acid section coding for the transporter domain (v).

2. Process according to Claim 1, characterized in that the autotransporter used has been derived from a genus of enterobacterioceae and is used in a host bacterium of a genus of enterobacterioceae.

3. Process according to Claim 1 or 2, characterized in that the transporter domain of the Aida protein from E.coli or a variant thereof is used.

4. Process according to Claim 1 or 2, characterized in that the transporter domain of the SepA protein from Shigella flexneri or a variant thereof is used.

5. Process according to Claim 1 or 2, characterized in that the transporter domain of the IcsA protein from Shigella flexneri or a variant thereof is used.

6. Process according to Claim 2, characterized in that the transporter domain of the Tsh protein from E.coli or a variant thereof is used.

7. Process according to Claim 2, characterized in that the transporter domain of the Ssp protein from Seratin marcescens or a variant thereof is used.

8. Process according to Claim 1, characterized in that the transporter domain of the Hsr protein from Helicobacter mustelae, of the Prn protein from Bordetella ssp., of the Hap protein from Haemophilus influenzae, of the BrkA protein from Bordetella pertussis, of the VacA protein from Helicobacter pylori or of one of the rickettsial proteins 190kDa cell surface protein, SpaP, rOmpB or SlpT, is used.

9. Process according to any of Claims 1-8, characterized in that one or more peptides, in particular peptides having a length of 4-50 amino acids, are presented.

10. Process according to any of Claims 1-8, characterized in that one or more eukaryotic polypeptides are presented.

11. Process according to Claim 10, characterized in that the passenger polypeptide is an antibody or an antigen-binding domain of an antibody, where antigen-binding domain refers to at least the region of an antibody molecule which is sufficient for specific binding of an antigen.

12. Process according to Claim 10, characterized in that the passenger polypeptide is the α chain of an MHC class II molecule.

13. Process according to Claim 10, characterized in that the passenger polypeptide is the β chain of an MHC class II molecule.

14. Process according to Claim 13, characterized in that the passenger polypeptide is the β chain of an MHC class II molecule, attached to whose N terminus are amino acids which, as peptide, are able to embed in the binding cavity of the functional MHC molecule.

15. Process according to any of Claims 1-14, characterized in that libraries of variant passenger peptides or polypeptides are produced, expressed in host cells and presented on the surface.

16. Process according to Claim 15, characterized in that the variant passenger peptides or polypeptides are presented in a constant context of a passenger polypeptide.

17. Process according to any of Claims 1-16, characterized in that a host bacterial cell presents different passenger peptides or polypeptides in each case connected to a transporter domain.

18. Process according to Claim 17, characterized in that different transporter domains are used in connection with different passenger peptides or polypeptides.

19. Process according to any of Claims 15-18, further comprising the step of selecting single passenger peptides or polypeptides from a library of variant peptides or polypeptides.

20. Process for the preparation of a variant population of surface-exposed peptides or polypeptides and for identification of the bacteria which carry peptides or polypeptides with a particular required property, where the process comprises the following steps:

(1) preparation of one or more fusion genes by cloning the coding sequence of a required passenger in frame with the coding sequence of the transporter domain of an autotransporter and of a signal peptide in at least one vector;

(2) variation of the passenger peptide or polypeptide by mutagenesis;

(3) introduction of the vector or vectors into host bacteria able to present the passenger or passengers stably on the surface;

(4) expression of the fusion gene or fusion genes in the host bacteria;

(5) cultivation of the bacteria to produce the passenger presented stably exposed on the surface or the passengers presented stably exposed on the surface;

(6) where appropriate selection of the bacteria which carry the passenger or passengers having the required properties on the surface, and

(7) where appropriate characterization of a binding partner for the passenger having the optimal properties.

21. Process according to Claim 20, where individual steps of the process can be omitted.

22. Process according to Claim 20, where the process is performed several times.

23. Process according to Claim 20, where the transporter domain AIDA-I or a variant thereof is used.

24. Process according to any of Claims 20-23, where the passenger protein present in the fusion protein is a peptide or polypeptide having an affinity for a binding partner, or is a ligand, a receptor, an antigen, a toxin-binding protein, a protein with enzymatic activity, a nucleic acid-binding protein, an inhibitor, a protein having chelator properties, an antibody or an antigen-binding domain of an antibody.

25. Process according to any of Claims 20-24, where the bacterium which presents a surface-exposed

passenger having a required binding affinity is identified by binding to an immobilized or/and labelled binding partner.

26. Process according to Claim 20, where the binding partner is modified so that it can be detected in a second step by a binding partner specific for the modification.

27. Process according to any of Claims 1-26, characterized in that passenger proteins or parts thereof are chemically or enzymatically modified on the bacterial surface.

28. Process according to Claim 27, characterized in that the modification is a non-covalent modification.

29. Process according to Claim 27, characterized in that the modification is a covalent modification.

30. Process according to Claim 29, characterized in that the modification is a glycosylation.

31. Process according to Claim 29, characterized in that the modification is a phosphorylation.

32. Process according to Claim 27, characterized in that the modification is a proteolysis.

33. Process according to Claim 32, characterized in that passenger proteins or parts thereof are selectively released from the bacterial surface by intrinsic or externally added proteases.

34. Process according to Claim 33, characterized in that passenger proteins or parts thereof are released by an intrinsic protease of the host cell, in particular OmpT protease, OmpK protease or protease X.

35. Process according to Claim 33, characterized in that passenger proteins or parts thereof are released by an externally added protease, in particular IgA protease, thrombin or factor X.

36. Recombinant vector on which is located, operatively linked to a promoter, a fused nucleic acid sequence comprising:

(i) a signal peptide-encoding nucleic acid section,

(ii) a nucleic acid section coding for the passenger peptide or/and passenger polypeptide to be presented,

(iii) where appropriate a nucleic acid section coding for a protease recognition site,

(iv) a nucleic acid section coding for a transmembrane linker and

(v) a nucleic acid section coding for a transporter domain of an autotransporter;

where the nucleic acid section (ii) is heterologous in relation to the nucleic acid section coding for the transporter domain (v).

37. Recombinant Gram-negative host bacterium, characterized in that it is transformed with a vector according to Claim 36.

38. Host bacterium according to Claim 37, characterized in that it is homologous in relation to the nucleic acid section coding for the transporter domain (v).

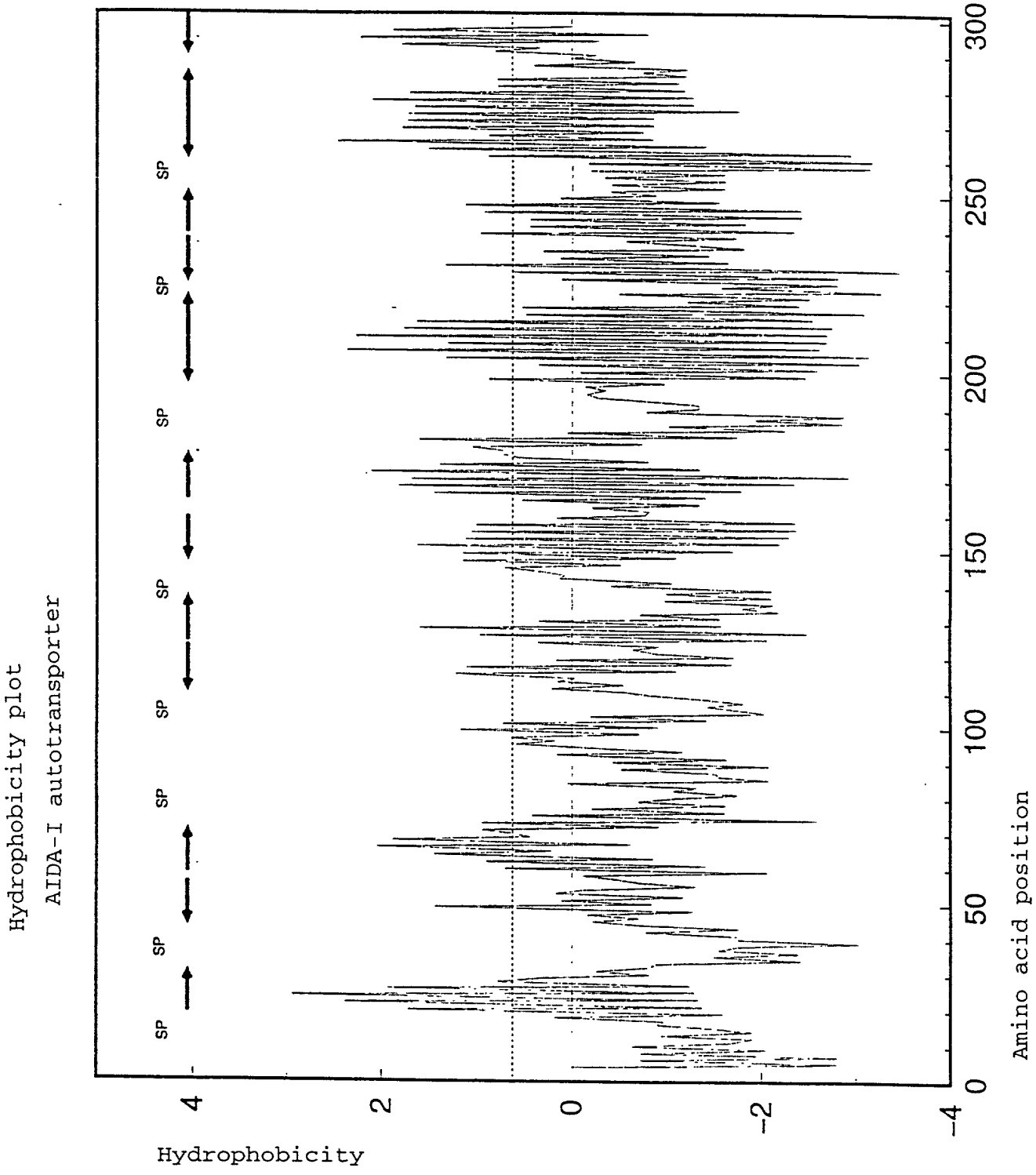
39. Host bacterium according to Claim 38, characterized in that it is an E.coli cell.

40. Host bacterium according to any of Claims 37-39, characterized in that the nucleic acid section (v) codes for the transporter domain of the AIDA protein or a variant thereof.

Abstract

The present invention relates to vectors, host-vector combinations and processes for preparing stable fusion proteins consisting of a carrier protein and a passenger protein, where expression of the fusion proteins leads to exposure of the passenger domains on the surface of bacterial cells, especially Escherichia coli cells. If required, the passenger domains can be released into the medium by proteases, for example by selected host factors such as, for example, OmpT.

Figure 1


Hydrophobicity plot
AIDA-I autotransporter
SP
SP
SP
SP
SP
SP
SP
SP
4
2
0
-2
-4
Hydrophobicity
0
50
100
150
200
250
300
Amino acid position

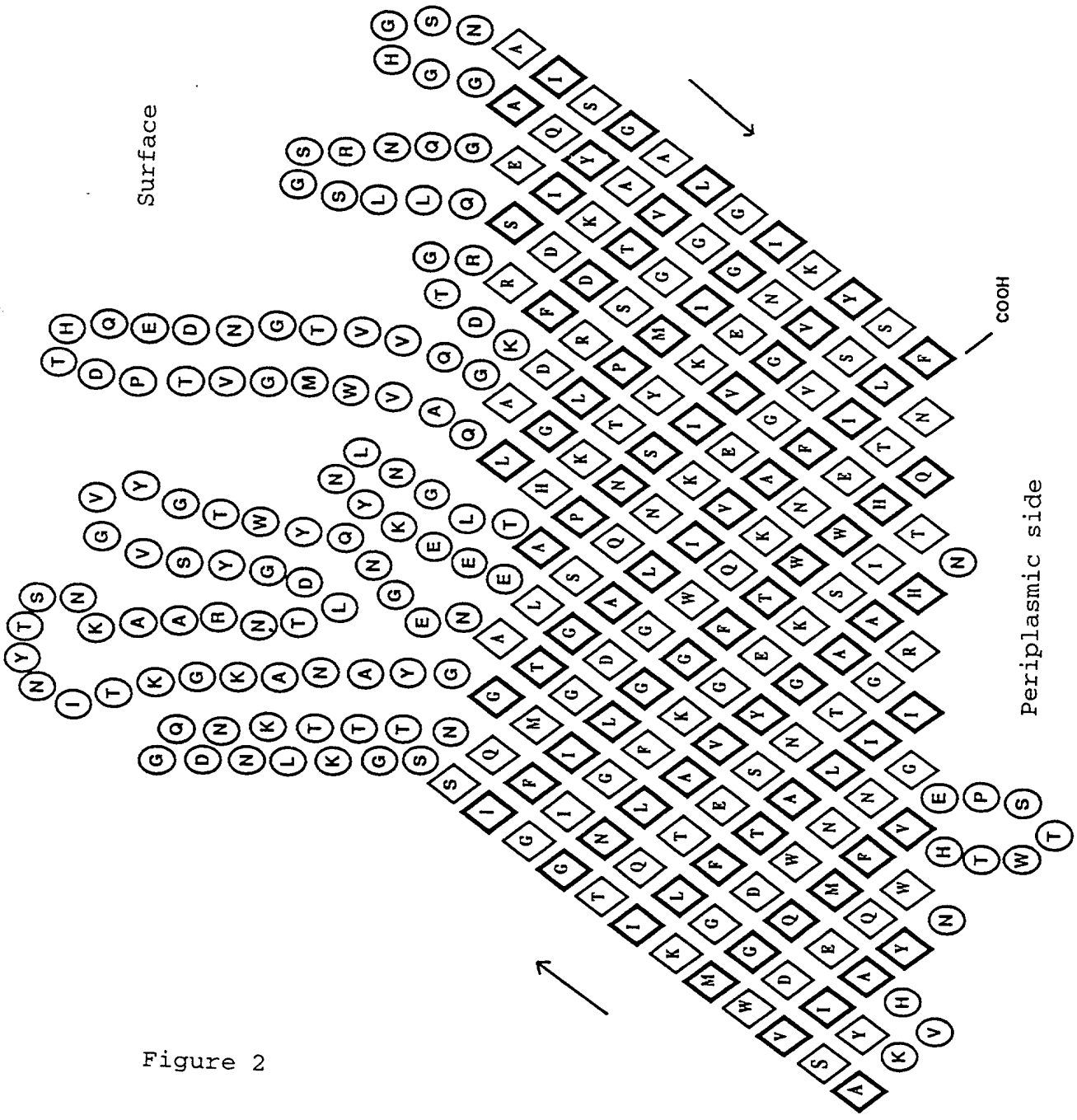


Figure 2

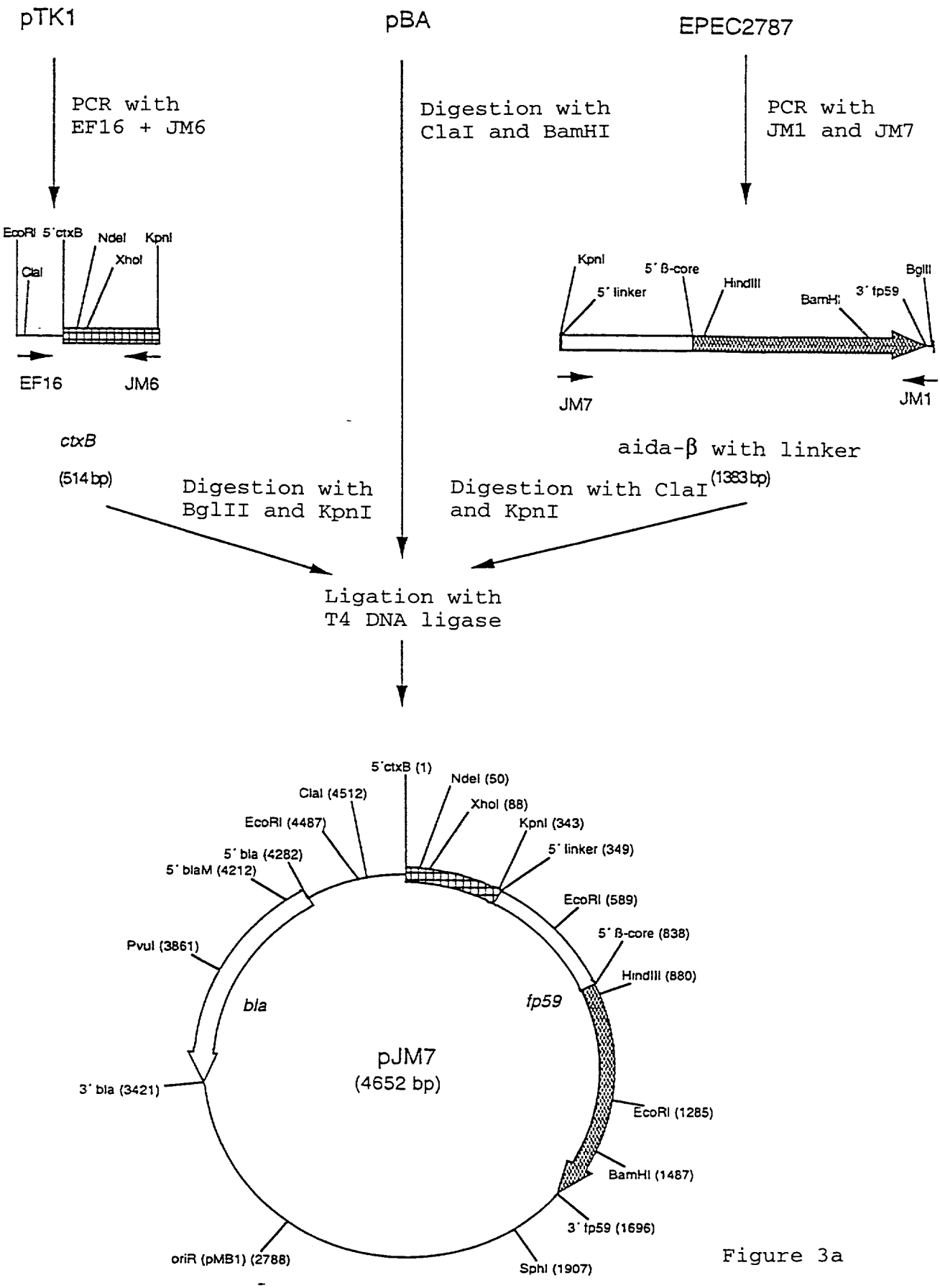

pTK1
pBA
EPEC2787
PCR with
EF16 + JM6
Digestion with
ClaI and BamHI
PCR with
JM1 and JM7
EcoRI 5'ctxB
NdeI
KpnI
XhoI
ClaI
EF16
JM6
KpnI
5' ß-core
HindIII
BglII
5' linker
BamHi
3' fp59
JM7
JM1
ctxB
(514 bp)
aida-β with linker
(1383 bp)
Digestion with
BglII and KpnI
Digestion with ClaI
and KpnI
Ligation with
T4 DNA ligase
5'ctxB (1)
NdeI (50)
ClaI (4512)
XhoI (88)
EcoRI (4487)
KpnI (343)
5' bla (4282)
5' linker (349)
5' blaM (4212)
EcoRI (589)
5' ß-core (838)
PvuI (3861)
HindIII (880)
bla
fp59
pJM7
(4652 bp)
3' bla (3421)
EcoRI (1285)
BamHI (1487)
3' fp59 (1696)
oriR (pMB1) (2788)
SphI (1907)

Figure 3a

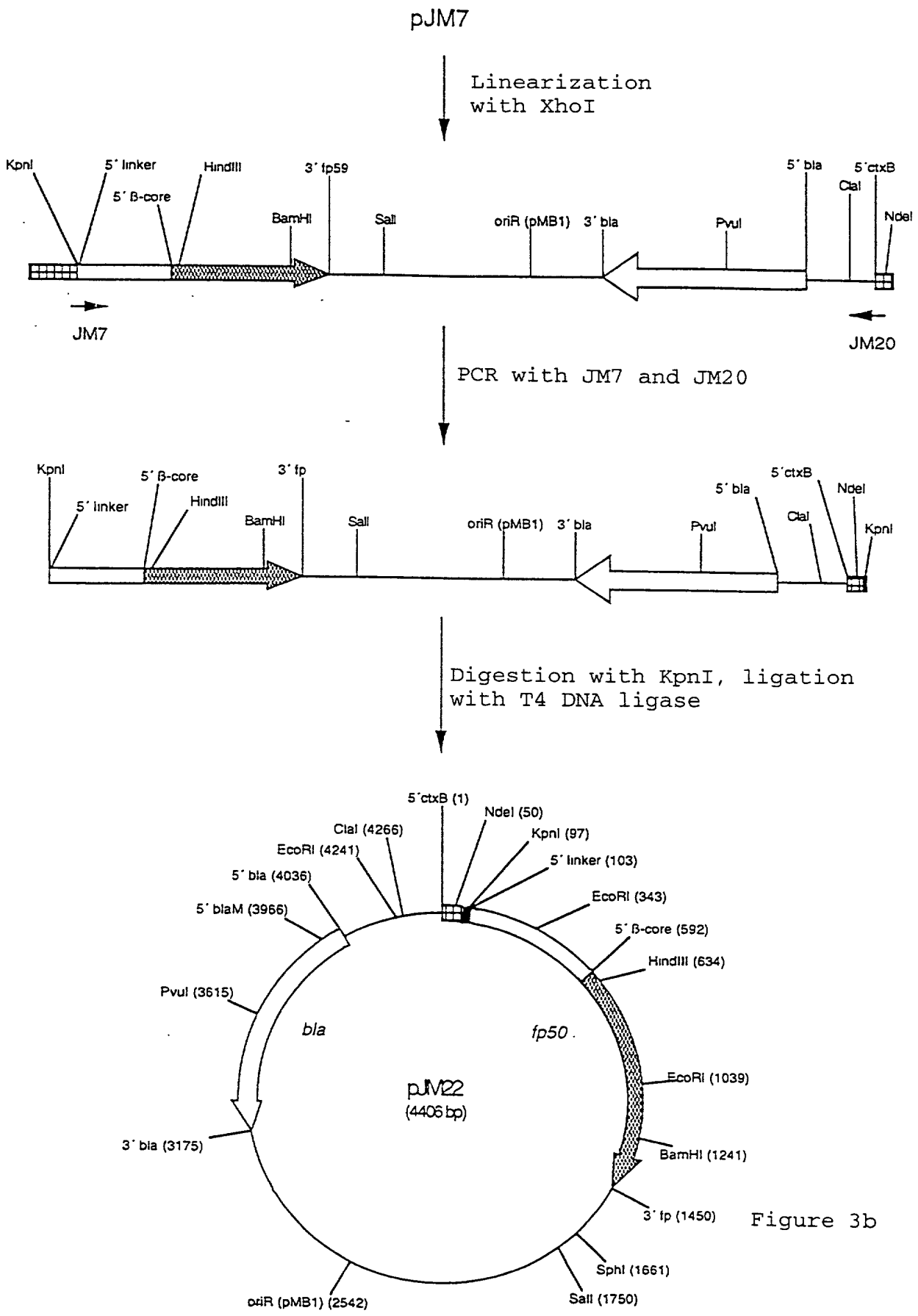
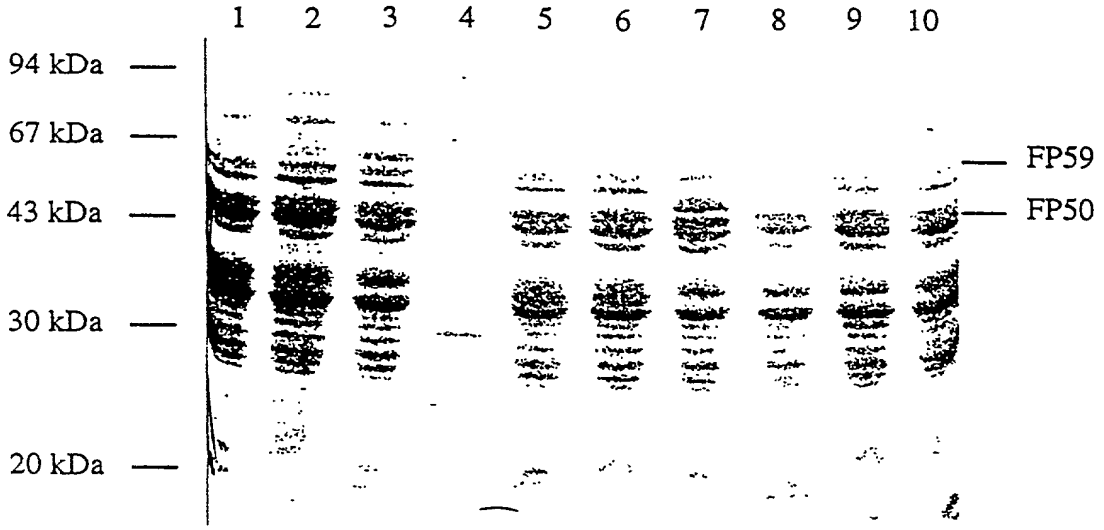

pJM7
Linearization with XhoI
KpnI
5' linker
HindIII
3' fp59
5' ß-core
BamHI
SalI
oriR (pMB1)
3' bla
PvuI
5' bla
5'ctxB
ClaI
NdeI
JM7
JM20
PCR with JM7 and JM20
KpnI
5' ß-core
3' fp
5' linker
HindIII
BamHI
SalI
oriR (pMB1)
3' bla
PvuI
5' bla
5'ctxB
NdeI
ClaI
KpnI
Digestion with KpnI, ligation with T4 DNA ligase
5'ctxB (1)
NdeI (50)
KpnI (97)
5' linker (103)
EcoRI (343)
5' ß-core (592)
HindIII (634)
EcoRI (1039)
BamHI (1241)
3' fp (1450)
SphI (1661)
SalI (1750)
oriR (pMB1) (2542)
3' bla (3175)
PvuI (3615)
5' blaM (3966)
5' bla (4036)
EcoRI (4241)
ClaI (4266)
bla
fp50
pJM22
(4406 bp)

Figure 3b

Figure 4a



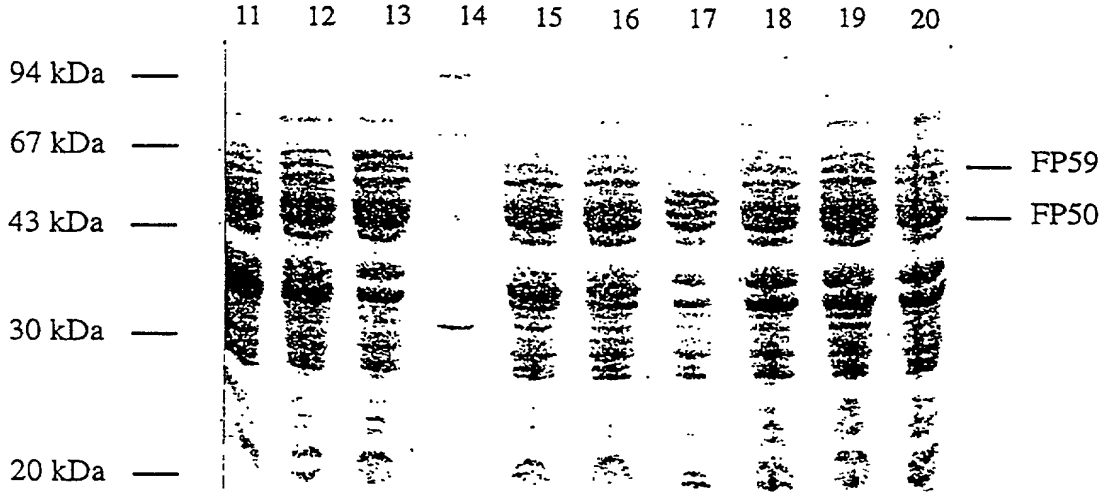


Figure 4b

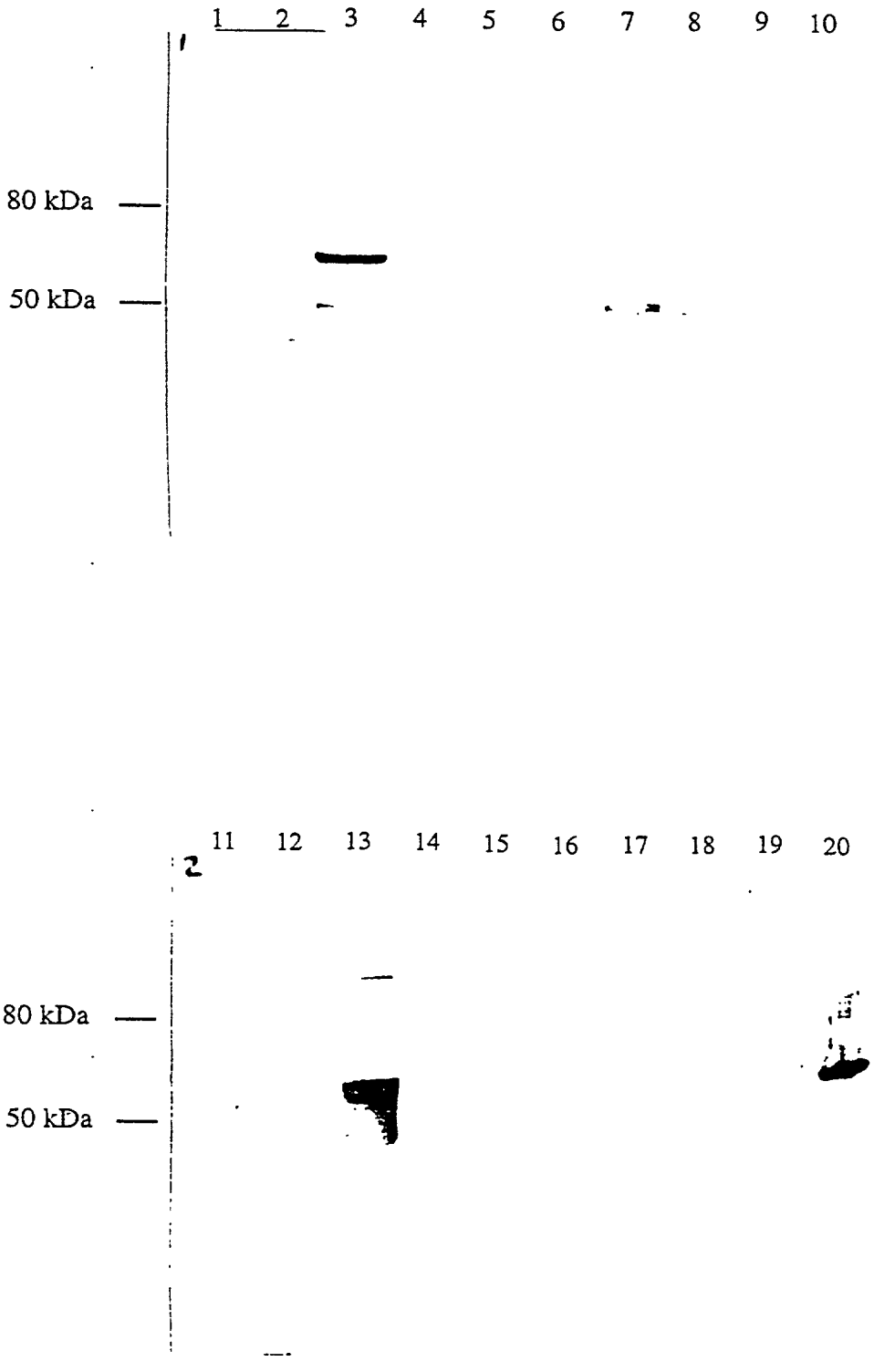
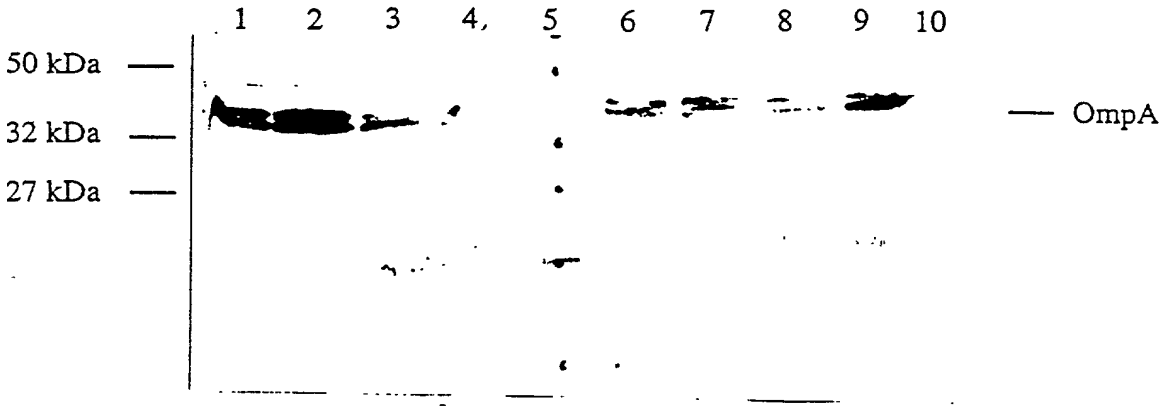

1 2 3 4 5 6 7 8 9 10
80 kDa
50 kDa
11 12 13 14 15 16 17 18 19 20
80 kDa
50 kDa

Figure 4c



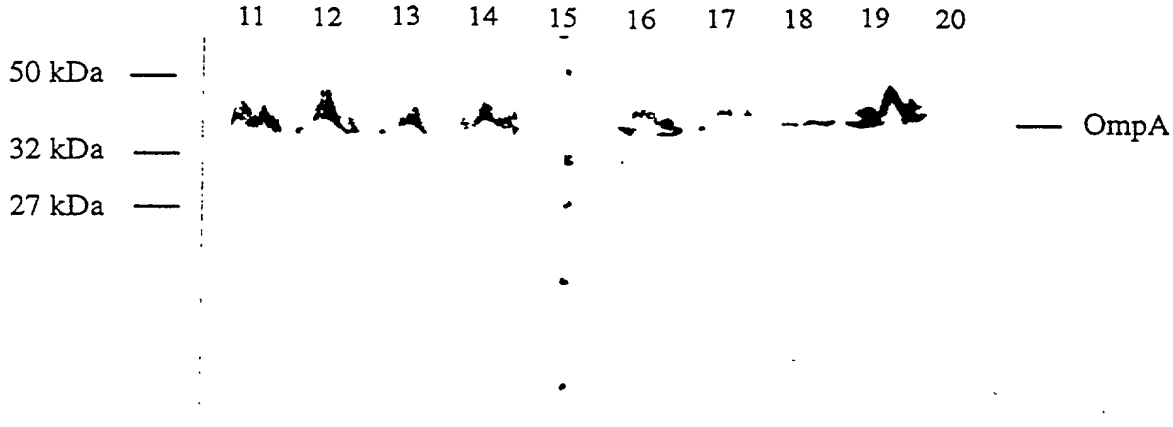
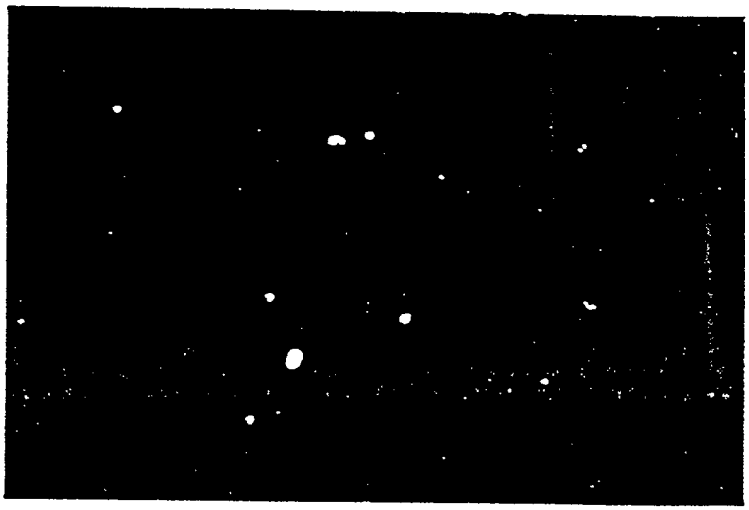


Figure 5

a) *E.coli* UT5600 pBA

b) *E.coli* UT5600 pTK1



c) *E.coli* UT5600 pJM7

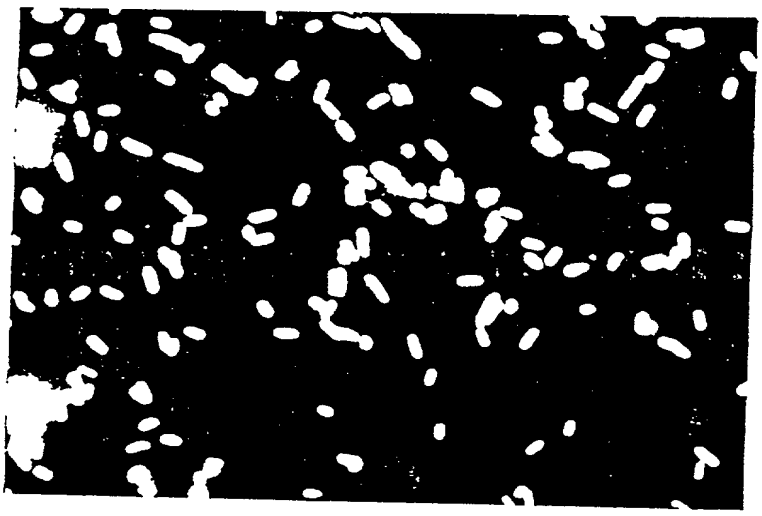
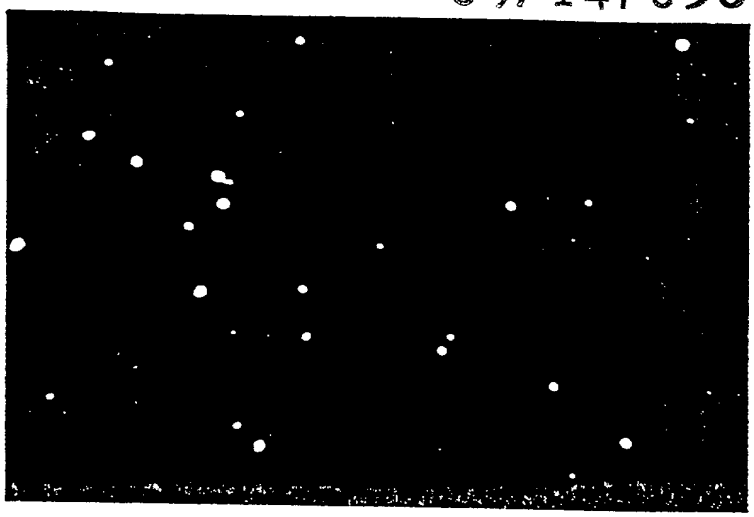


Figure 5

d) *E.coli* UT5600 pJM22



e) *E.coli* UT5600 pTK61

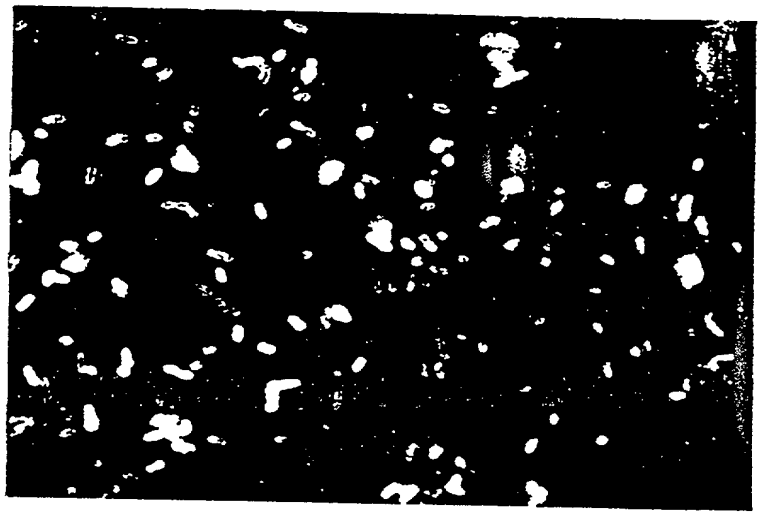


Figure 6

DNA sequences of the oligonucleotides used

Name	Use 1)	Length (bp)	Sequence (5'-3')
EF16	PCR (+)	36	TGTAAAACGACGGCCAGTATCACGAGG CCCTTTCGT
JM1	PCR (-)	27	GGAAGATCTGCCTCAGAAATGAGGGCC
JM6	PCR (-)	30	CATGGTACCAGGCGTTTTATTATTCCCT AC
JM7	PCR (+)	30	CGGGGTACCCTTAATCCTACAAAAGAA AGT
JM20	PCR (+)	44	AAGGGTACCTTTGAAATACTCCGGAGTA ATATTTTTGAGGTGTTC

1)
(+) and (-) relate to the coding (+) and the DNA strand complementary thereto (-).

Figure 7

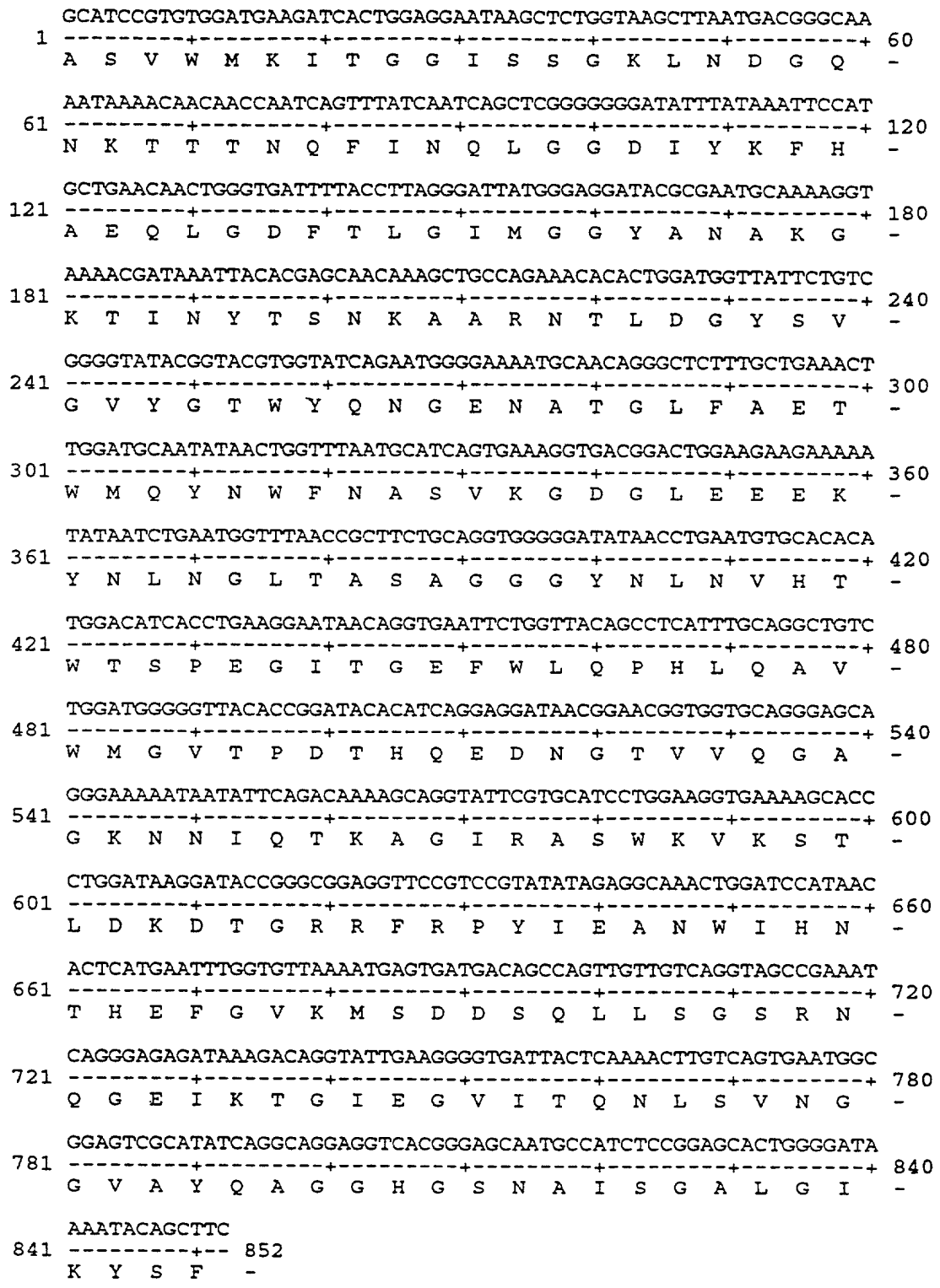

GCATCCGTGTGGATGAAGATCACTGGAGGAATAAGCTCTGGTAAGCTTAATGACGGGCAA
1
60
A S V W M K I T G G I S S G K L N D G Q -
AATAAAACAACAACCAATCAGTTTATCAATCAGCTCGGGGGGGATATTTATAAATTCCAT
61
120
N K T T T N Q F I N Q L G G D I Y K F H -
GCTGAACAACTGGGTGATTTTACCTTAGGGATTATGGGAGGATACGCGAATGCAAAAGGT
121
180
A E Q L G D F T L G I M G G Y A N A K G -
AAAACGATAAATTACACGAGCAACAAAGCTGCCAGAAACACACTGGATGGTTATTCTGTC
181
240
K T I N Y T S N K A A R N T L D G Y S V -
GGGGTATACGGTACGTGGTATCAGAATGGGGAAAATGCAACAGGGCTCTTTGCTGAAACT
241
300
G V Y G T W Y Q N G E N A T G L F A E T -
TGGATGCAATATAACTGGTTTAATGCATCAGTGAAAGGTGACGGACTGGAAGAAGAAAAA
301
360
W M Q Y N W F N A S V K G D G L E E E K -
TATAATCTGAATGGTTTAACCGCTTCTGCAGGTGGGGGATATAACCTGAATGTGCACACA
361
420
Y N L N G L T A S A G G G Y N L N V H T -
TGGACATCACCTGAAGGAATAACAGGTGAATTCTGGTTACAGCCTCATTTGCAGGCTGTC
421
480
W T S P E G I T G E F W L Q P H L Q A V -
TGGATGGGGGTTACACCGGATACACATCAGGAGGATAACGGAACGGTGGTGCAGGGAGCA
481
540
W M G V T P D T H Q E D N G T V V Q G A -
GGGAAAAATAATATTCAGACAAAAGCAGGTATTCGTGCATCCTGGAAGGTGAAAAGCACC
541
600
G K N N I Q T K A G I R A S W K V K S T -
CTGGATAAGGATACCGGGCGGAGGTTCCGTCCGTATATAGAGGCAAACTGGATCCATAAC
601
660
L D K D T G R R F R P Y I E A N W I H N -
ACTCATGAATTTGGTGTTAAAATGAGTGATGACAGCCAGTTGTTGTCAGGTAGCCGAAAT
661
720
T H E F G V K M S D D S Q L L S G S R N -
CAGGGAGAGATAAAGACAGGTATTGAAGGGGTGATTACTCAAAACTTGTCAGTGAATGGC
721
780
Q G E I K T G I E G V I T Q N L S V N G -
GGAGTCGCATATCAGGCAGGAGGTCACGGGAGCAATGCCATCTCCGGAGCACTGGGGATA
781
840
G V A Y Q A G G H G S N A I S G A L G I -
AAATACAGCTTC
841
852
K Y S F -

Figure 8

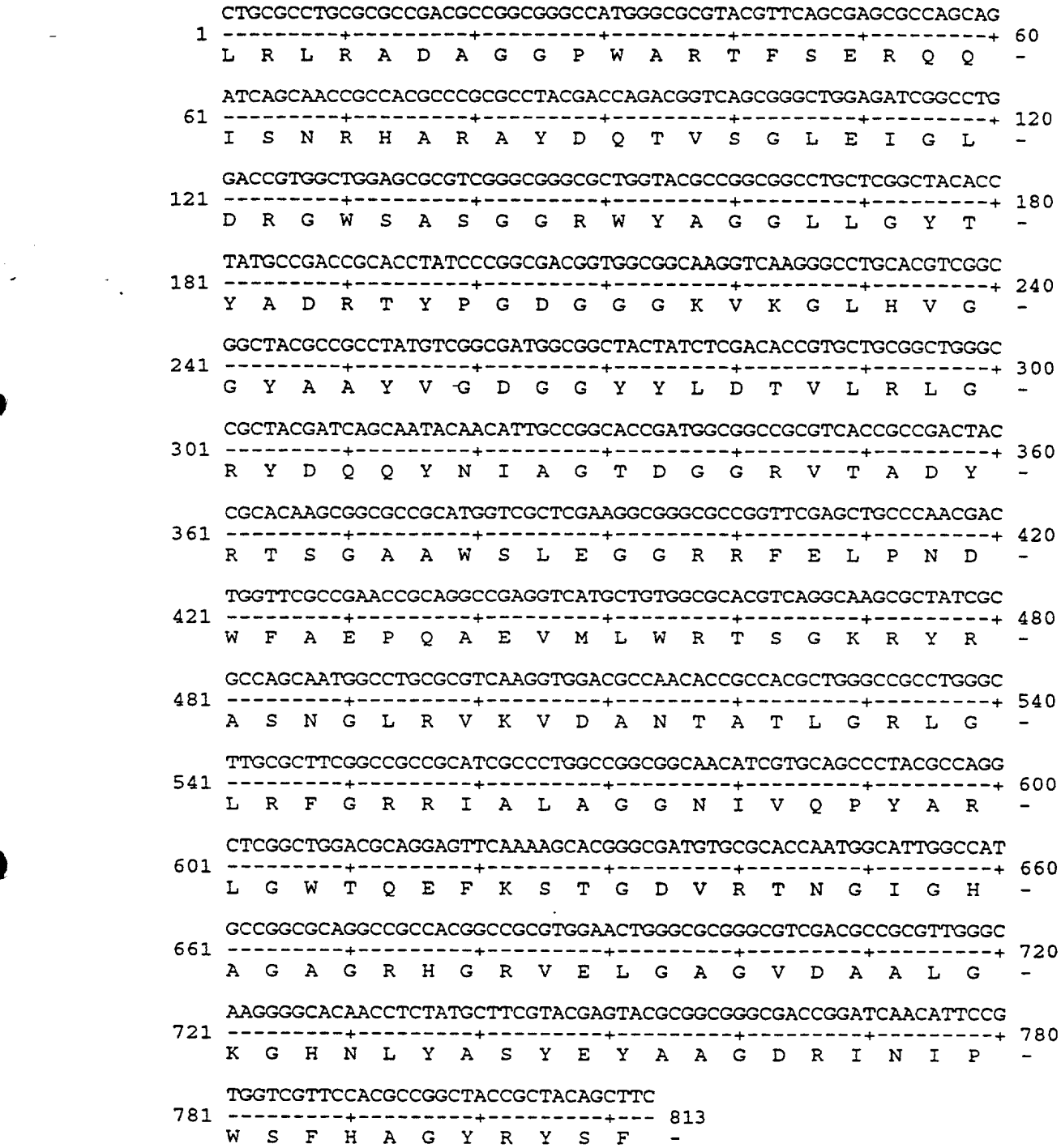

CTGCGCCTGCGCGCCGACGCCGGCGGGCCATGGGCGCGTACGTTCAGCGAGCGCCAGCAG
1 60
L R L R A D A G G P W A R T F S E R Q Q -
ATCAGCAACCGCCACGCCCGCGCCTACGACCAGACGGTCAGCGGGCTGGAGATCGGCCTG
61 120
I S N R H A R A Y D Q T V S G L E I G L -
GACCGTGGCTGGAGCGCGTCGGGCGGGCGCTGGTACGCCGGCGGCCTGCTCGGCTACACC
121 180
D R G W S A S G G R W Y A G G L L G Y T -
TATGCCGACCGCACCTATCCCGGCGACGGTGGCGGCAAGGTCAAGGGCCTGCACGTCGGC
181 240
Y A D R T Y P G D G G G K V K G L H V G -
GGCTACGCCGCCTATGTCGGCGATGGCGGCTACTATCTCGACACCGTGCTGCGGCTGGGC
241 300
G Y A A Y V G D G G Y Y L D T V L R L G -
CGCTACGATCAGCAATACAACATTGCCGGCACCGATGGCGGCCGCGTCACCGCCGACTAC
301 360
R Y D Q Q Y N I A G T D G G R V T A D Y -
CGCACAAGCGGCGCCGCATGGTCGCTCGAAGGCGGGCGCCGGTTCGAGCTGCCCAACGAC
361 420
R T S G A A W S L E G G R R F E L P N D -
TGGTTCGCCGAACCGCAGGCCGAGGTCATGCTGTGGCGCACGTCAGGCAAGCGCTATCGC
421 480
W F A E P Q A E V M L W R T S G K R Y R -
GCCAGCAATGGCCTGCGCGTCAAGGTGGACGCCAACACCGCCACGCTGGGCCGCCTGGGC
481 540
A S N G L R V K V D A N T A T L G R L G -
TTGCGCTTCGGCCGCCGCATCGCCCTGGCCGGCGGCAACATCGTGCAGCCCTACGCCAGG
541 600
L R F G R R I A L A G G N I V Q P Y A R -
CTCGGCTGGACGCAGGAGTTCAAAAGCACGGGCGATGTGCGCACCAATGGCATTGGCCAT
601 660
L G W T Q E F K S T G D V R T N G I G H -
GCCGGCGCAGGCCGCCACGGCCGCGTGGAACTGGGCGCGGGCGTCGACGCCGCGTTGGGC
661 720
A G A G R H G R V E L G A G V D A A L G -
AAGGGGCACAACCTCTATGCTTCGTACGAGTACGCGGCGGGCGACCGGATCAACATTCCG
721 780
K G H N L Y A S Y E Y A A G D R I N I P -
TGGTCGTTCCACGCCGGCTACCGCTACAGCTTC
781 813
W S F H A G Y R Y S F -

Figure 9

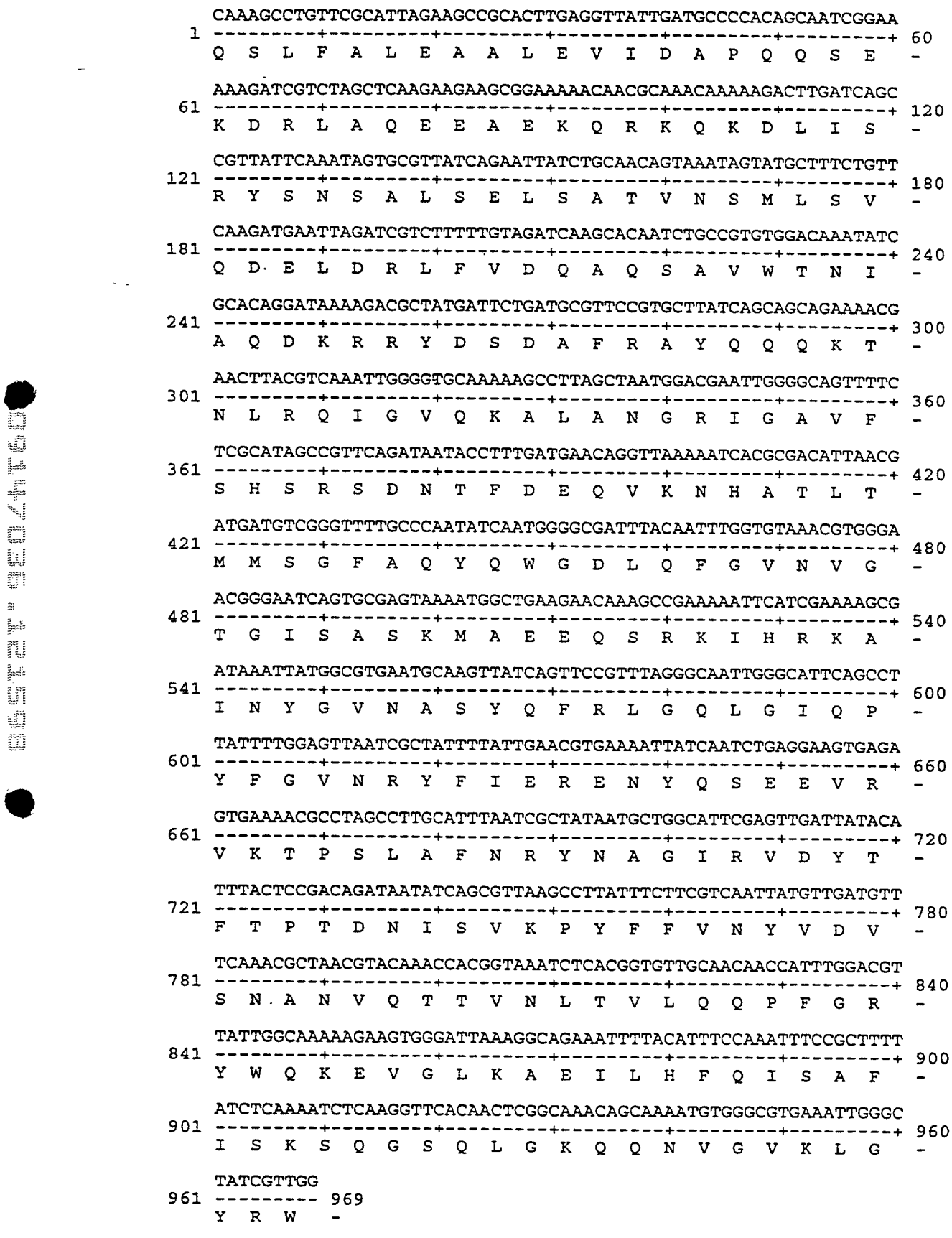


Figure 10

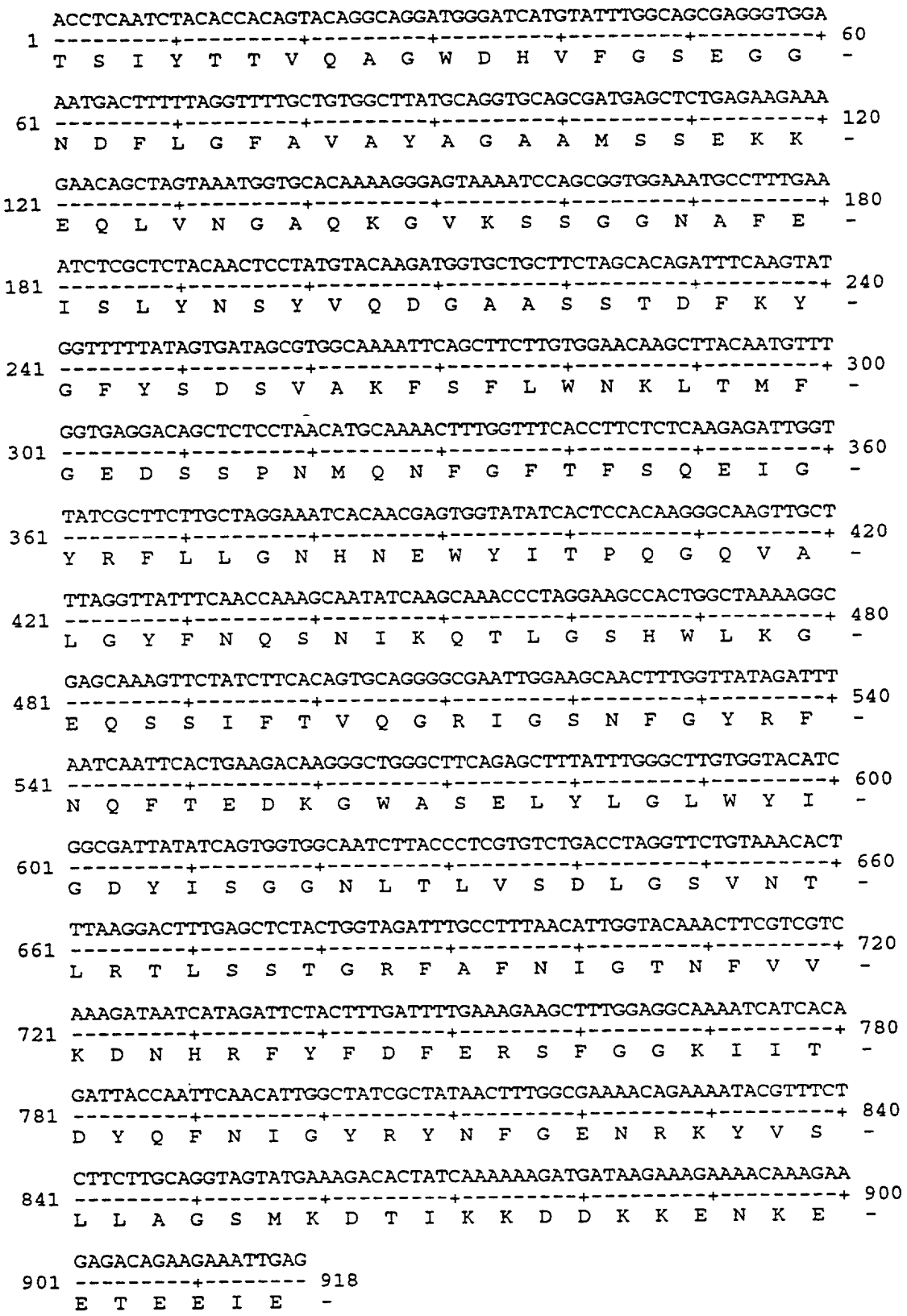

ACCTCAATCTACACCACAGTACAGGCAGGATGGGATCATGTATTTGGCAGCGAGGGTGGA
1 60
T S I Y T T V Q A G W D H V F G S E G G -
AATGACTTTTTAGGTTTTGCTGTGGCTTATGCAGGTGCAGCGATGAGCTCTGAGAAGAAA
61 120
N D F L G F A V A Y A G A A M S S E K K -
GAACAGCTAGTAAATGGTGCACAAAAGGGAGTAAAATCCAGCGGTGGAAATGCCTTTGAA
121 180
E Q L V N G A Q K G V K S S G G N A F E -
ATCTCGCTCTACAACTCCTATGTACAAGATGGTGCTGCTTCTAGCACAGATTTCAAGTAT
181 240
I S L Y N S Y V Q D G A A S S T D F K Y -
GGTTTTTATAGTGATAGCGTGGCAAAATTCAGCTTCTTGTGGAACAAGCTTACAATGTTT
241 300
G F Y S D S V A K F S F L W N K L T M F -
GGTGAGGACAGCTCTCCTAACATGCAAAACTTTGGTTTCACCTTCTCTCAAGAGATTGGT
301 360
G E D S S P N M Q N F G F T F S Q E I G -
TATCGCTTCTTGCTAGGAAATCACAACGAGTGGTATATCACTCCACAAGGGCAAGTTGCT
361 420
Y R F L L G N H N E W Y I T P Q G Q V A -
TTAGGTTATTTCAACCAAAGCAATATCAAGCAAACCCTAGGAAGCCACTGGCTAAAAGGC
421 480
L G Y F N Q S N I K Q T L G S H W L K G -
GAGCAAAGTTCTATCTTCACAGTGCAGGGGCGAATTGGAAGCAACTTTGGTTATAGATTT
481 540
E Q S S I F T V Q G R I G S N F G Y R F -
AATCAATTCACTGAAGACAAGGGCTGGGCTTCAGAGCTTTATTTGGGCTTGTGGTACATC
541 600
N Q F T E D K G W A S E L Y L G L W Y I -
GGCGATTATATCAGTGGTGGCAATCTTACCCTCGTGTCTGACCTAGGTTCTGTAAACACT
601 660
G D Y I S G G N L T L V S D L G S V N T -
TTAAGGACTTTGAGCTCTACTGGTAGATTTGCCTTTAACATTGGTACAAACTTCGTCGTC
661 720
L R T L S S T G R F A F N I G T N F V V -
AAAGATAATCATAGATTCTACTTTGATTTTGAAAGAAGCTTTGGAGGCAAAATCATCACA
721 780
K D N H R F Y F D F E R S F G G K I I T -
GATTACCAATTCAACATTGGCTATCGCTATAACTTTGGCGAAAACAGAAAATACGTTTCT
781 840
D Y Q F N I G Y R Y N F G E N R K Y V S -
CTTCTTGCAGGTAGTATGAAAGACACTATCAAAAAAGATGATAAGAAAGAAAACAAAGAA
841 900
L L A G S M K D T I K K D D K K E N K E -
GAGACAGAAGAAATTGAG
901 918
E T E E I E -

Figure 11

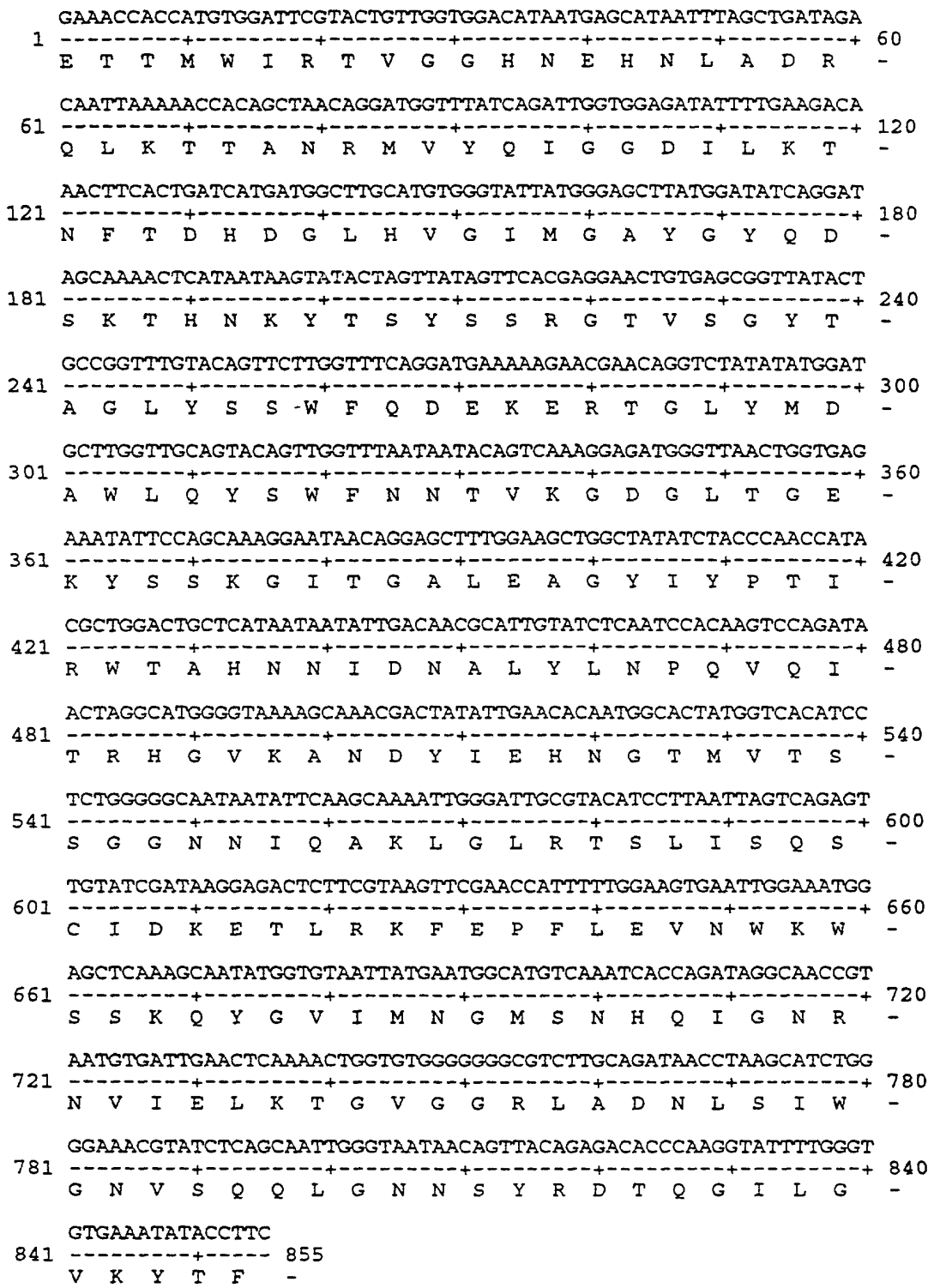


Figure 12

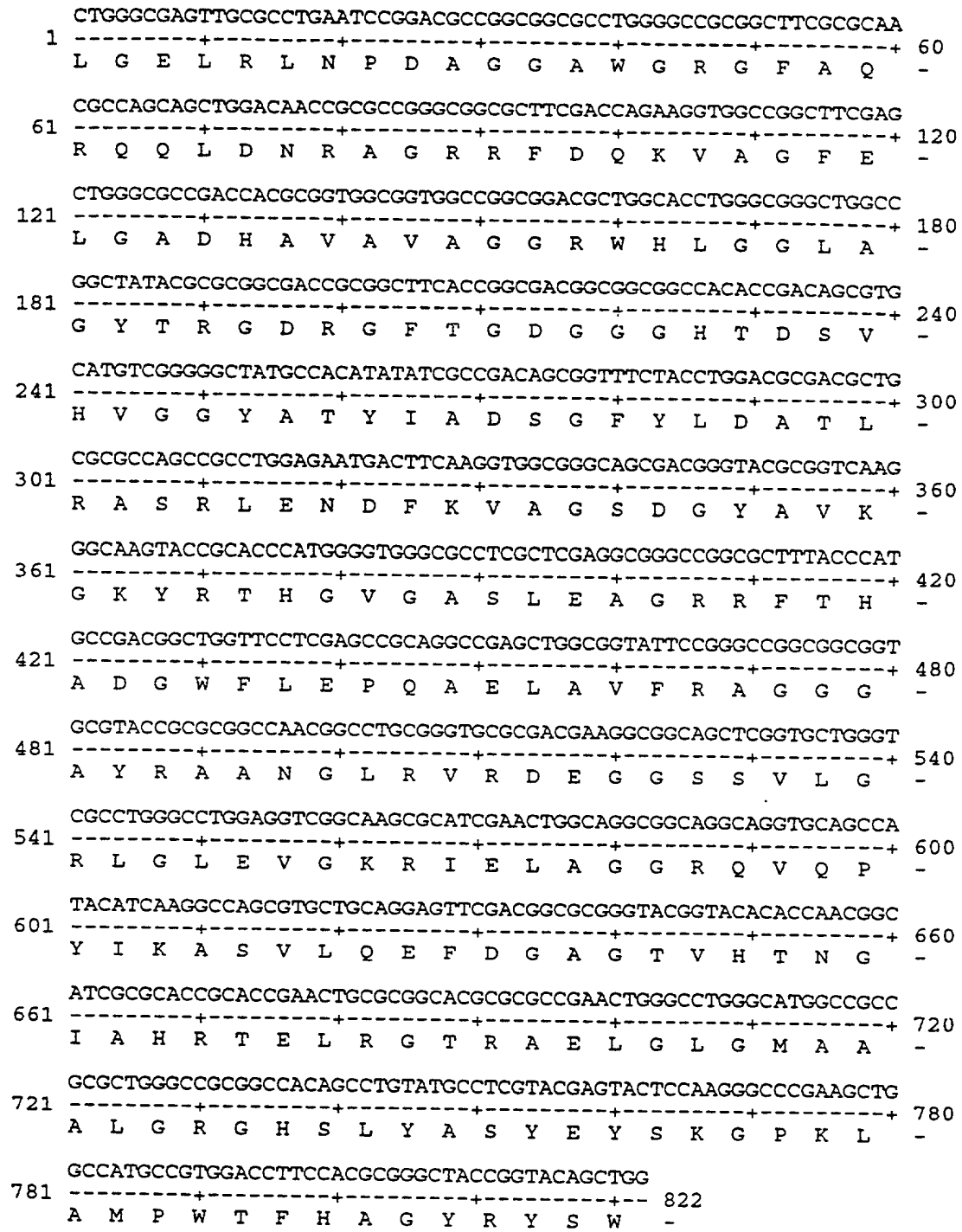

CTGGGCGAGTTGCGCCTGAATCCGGACGCCGGCGGCGCCTGGGGCCGCGGCTTCGCGCAA
L G E L R L N P D A G G A W G R G F A Q
CGCCAGCAGCTGGACAACCGCGCCGGGCGGCGCTTCGACCAGAAGGTGGCCGGCTTCGAG
R Q Q L D N R A G R R F D Q K V A G F E
CTGGGCGCCGACCACGCGGTGGCGGTGGCCGGCGGACGCTGGCACCTGGGCGGGCTGGCC
L G A D H A V A V A G G R W H L G G L A
GGCTATACGCGCGGCGACCGCGGCTTCACCGGCGACGGCGGCGGCCACACCGACAGCGTG
G Y T R G D R G F T G D G G G H T D S V
CATGTCGGGGGCTATGCCACATATATCGCCGACAGCGGTTTCTACCTGGACGCGACGCTG
H V G G Y A T Y I A D S G F Y L D A T L
CGCGCCAGCCGCCTGGAGAATGACTTCAAGGTGGCGGGCAGCGACGGGTACGCGGTCAAG
R A S R L E N D F K V A G S D G Y A V K
GGCAAGTACCGCACCCATGGGGTGGGCGCCTCGCTCGAGGCGGGCCGGCGCTTTACCCAT
G K Y R T H G V G A S L E A G R R F T H
GCCGACGGCTGGTTCCTCGAGCCGCAGGCCGAGCTGGCGGTATTCCGGGCCGGCGGCGGT
A D G W F L E P Q A E L A V F R A G G G
GCGTACCGCGCGGCCAACGGCCTGCGGGTGCGCGACGAAGGCGGCAGCTCGGTGCTGGGT
A Y R A A N G L R V R D E G G S S V L G
CGCCTGGGCCTGGAGGTCGGCAAGCGCATCGAACTGGCAGGCGGCAGGCAGGTGCAGCCA
R L G L E V G K R I E L A G G R Q V Q P
TACATCAAGGCCAGCGTGCTGCAGGAGTTCGACGGCGCGGGTACGGTACACACCAACGGC
Y I K A S V L Q E F D G A G T V H T N G
ATCGCGCACCGCACCGAACTGCGCGGCACGCGCGCCGAACTGGGCCTGGGCATGGCCGCC
I A H R T E L R G T R A E L G L G M A A
GCGCTGGGCCGCGGCCACAGCCTGTATGCCTCGTACGAGTACTCCAAGGGCCCGAAGCTG
A L G R G H S L Y A S Y E Y S K G P K L
GCCATGCCGTGGACCTTCCACGCGGGCTACCGGTACAGCTGG
A M P W T F H A G Y R Y S W
1 60 61 120 121 180 181 240 241 300 301 360 361 420 421 480 481 540 541 600 601 660 661 720 721 780 781 822

Figure 13

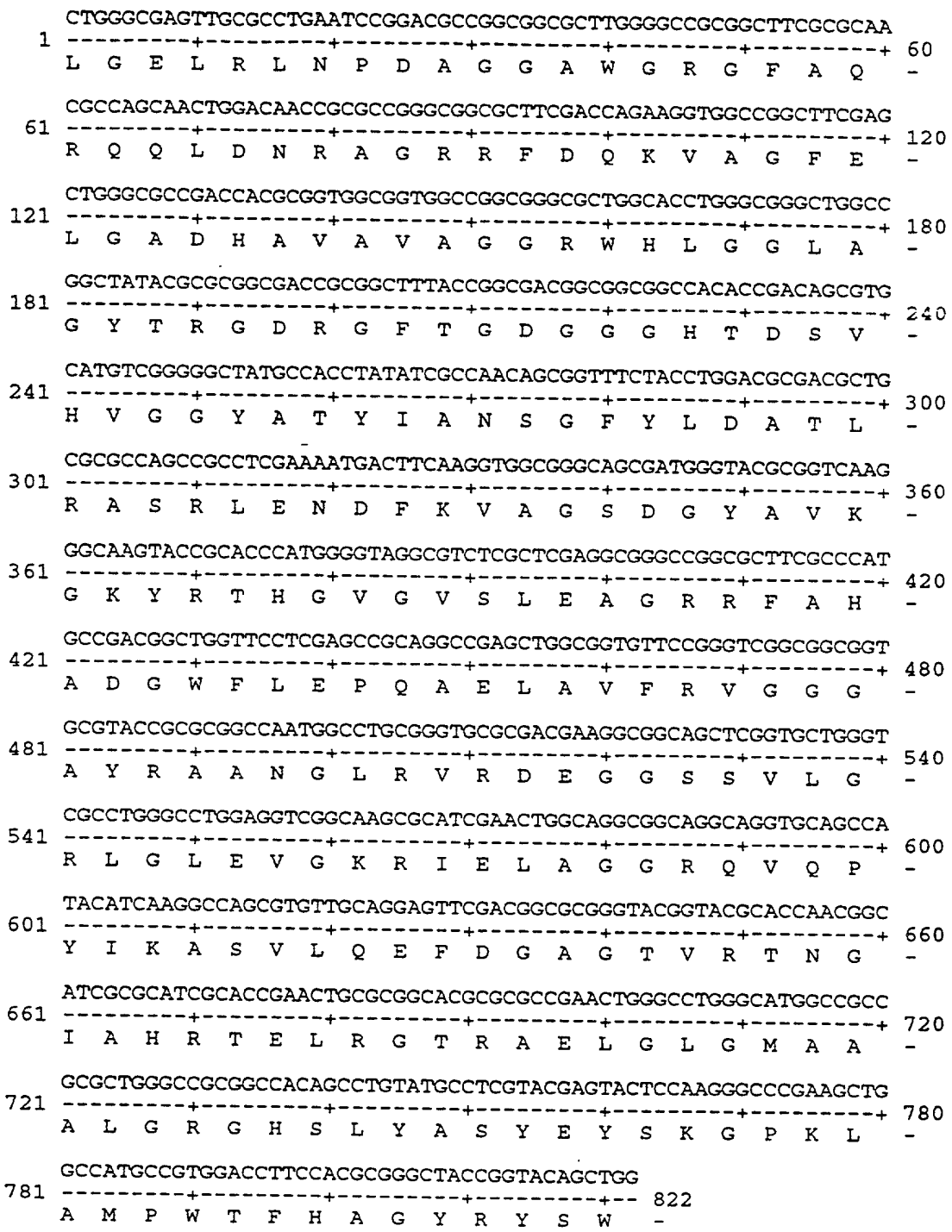

CTGGGCGAGTTGCGCCTGAATCCGGACGCCGGCGGCGCTTGGGGCCGCGGCTTCGCGCAA
1
L G E L R L N P D A G G A W G R G F A Q
60
CGCCAGCAACTGGACAACCGCGCCGGGCGGCGCTTCGACCAGAAGGTGGCCGGCTTCGAG
61
R Q Q L D N R A G R R F D Q K V A G F E
120
CTGGGCGCCGACCACGCGGTGGCGGTGGCCGGCGGGCGCTGGCACCTGGGCGGGCTGGCC
121
L G A D H A V A V A G G R W H L G G L A
180
GGCTATACGCGCGGCGACCGCGGCTTTACCGGCGACGGCGGCGGCCACACCGACAGCGTG
181
G Y T R G D R G F T G D G G G H T D S V
240
CATGTCGGGGGCTATGCCACCTATATCGCCAACAGCGGTTTCTACCTGGACGCGACGCTG
241
H V G G Y A T Y I A N S G F Y L D A T L
300
CGCGCCAGCCGCCTCGAAAATGACTTCAAGGTGGCGGGCAGCGATGGGTACGCGGTCAAG
301
R A S R L E N D F K V A G S D G Y A V K
360
GGCAAGTACCGCACCCATGGGGTAGGCGTCTCGCTCGAGGCGGGCCGGCGCTTCGCCCAT
361
G K Y R T H G V G V S L E A G R R F A H
420
GCCGACGGCTGGTTCCTCGAGCCGCAGGCCGAGCTGGCGGTGTTCCGGGTCGGCGGCGGT
421
A D G W F L E P Q A E L A V F R V G G G
480
GCGTACCGCGCGGCCAATGGCCTGCGGGTGCGCGACGAAGGCGGCAGCTCGGTGCTGGGT
481
A Y R A A N G L R V R D E G G S S V L G
540
CGCCTGGGCCTGGAGGTCGGCAAGCGCATCGAACTGGCAGGCGGCAGGCAGGTGCAGCCA
541
R L G L E V G K R I E L A G G R Q V Q P
600
TACATCAAGGCCAGCGTGTTGCAGGAGTTCGACGGCGCGGGTACGGTACGCACCAACGGC
601
Y I K A S V L Q E F D G A G T V R T N G
660
ATCGCGCATCGCACCGAACTGCGCGGCACGCGCGCCGAACTGGGCCTGGGCATGGCCGCC
661
I A H R T E L R G T R A E L G L G M A A
720
GCGCTGGGCCGCGGCCACAGCCTGTATGCCTCGTACGAGTACTCCAAGGGCCCGAAGCTG
721
A L G R G H S L Y A S Y E Y S K G P K L
780
GCCATGCCGTGGACCTTCCACGCGGGCTACCGGTACAGCTGG
781
A M P W T F H A G Y R Y S W
822

Figure 14

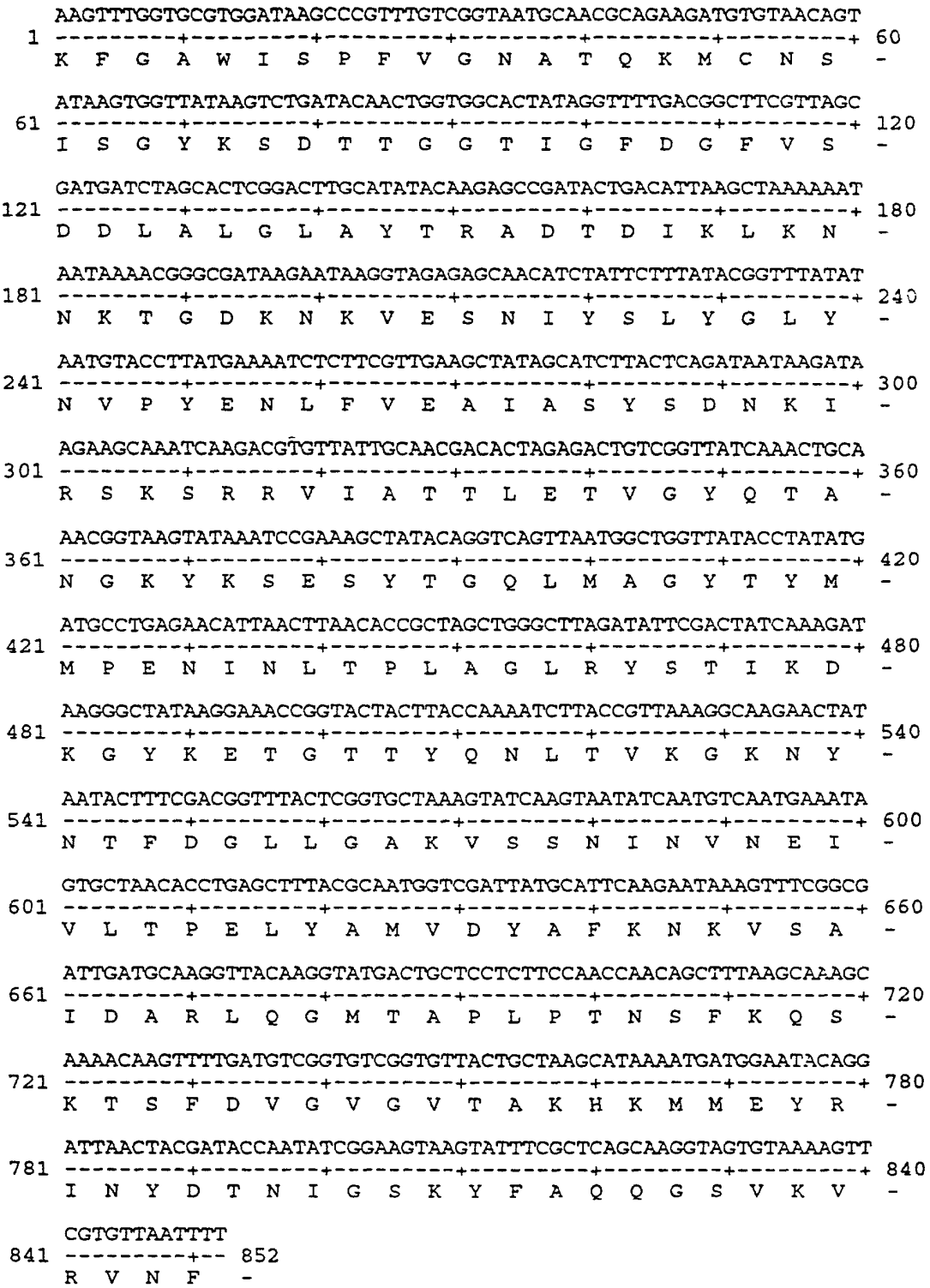


Figure 15

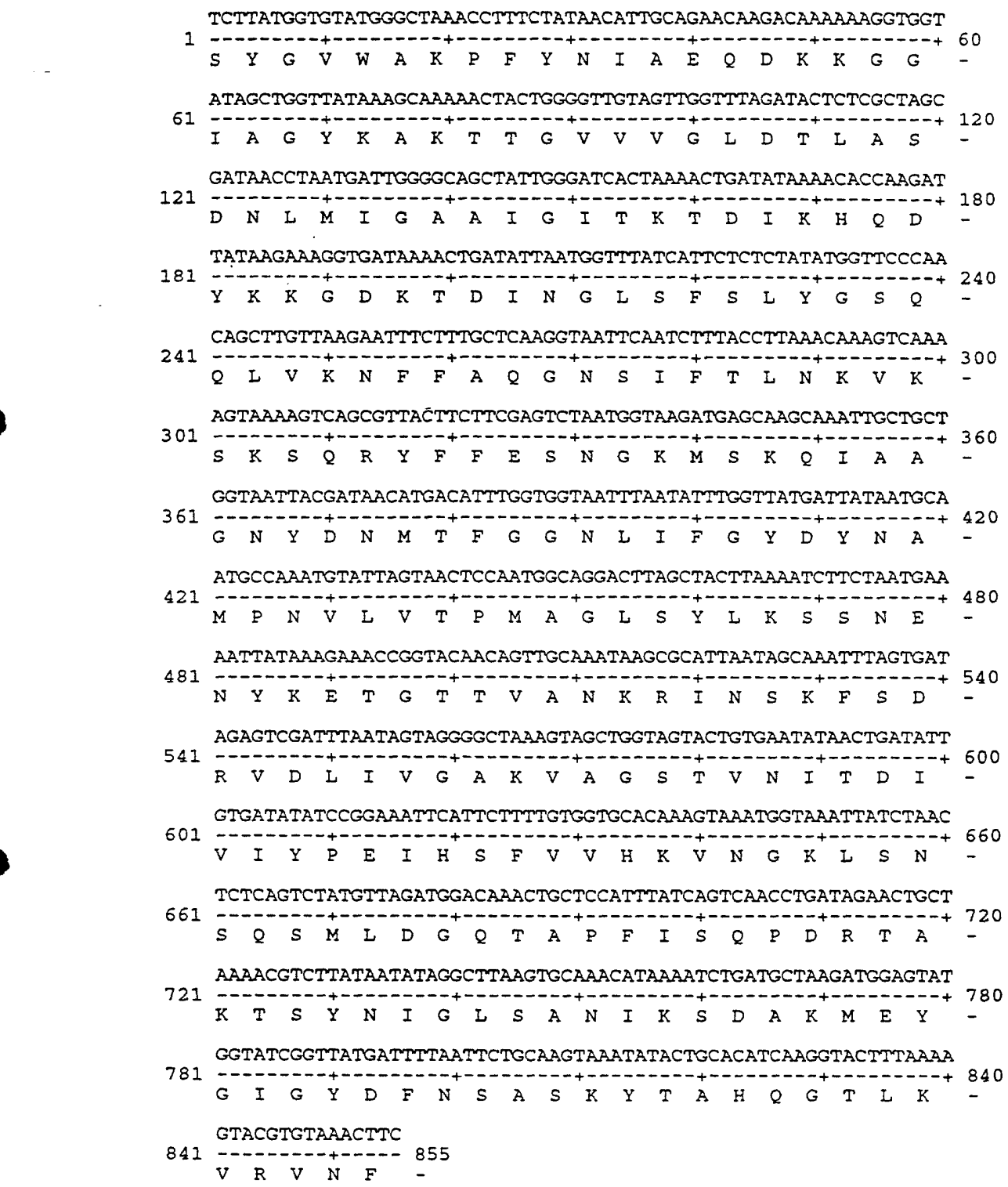

TCTTATGGTGTATGGGCTAAACCTTTCTATAACATTGCAGAACAAGACAAAAAAGGTGGT
S Y G V W A K P F Y N I A E Q D K K G G
ATAGCTGGTTATAAAGCAAAAACTACTGGGGTTGTAGTTGGTTTAGATACTCTCGCTAGC
I A G Y K A K T T G V V V G L D T L A S
GATAACCTAATGATTGGGGCAGCTATTGGGATCACTAAAACTGATATAAAACACCAAGAT
D N L M I G A A I G I T K T D I K H Q D
TATAAGAAAGGTGATAAAACTGATATTAATGGTTTATCATTCTCTCTATATGGTTCCCAA
Y K K G D K T D I N G L S F S L Y G S Q
CAGCTTGTTAAGAATTTCTTTGCTCAAGGTAATTCAATCTTTACCTTAAACAAAGTCAAA
Q L V K N F F A Q G N S I F T L N K V K
AGTAAAAGTCAGCGTTACTTCTTCGAGTCTAATGGTAAGATGAGCAAGCAAATTGCTGCT
S K S Q R Y F F E S N G K M S K Q I A A
GGTAATTACGATAACATGACATTTGGTGGTAATTTAATATTTGGTTATGATTATAATGCA
G N Y D N M T F G G N L I F G Y D Y N A
ATGCCAAATGTATTAGTAACTCCAATGGCAGGACTTAGCTACTTAAAATCTTCTAATGAA
M P N V L V T P M A G L S Y L K S S N E
AATTATAAAGAAACCGGTACAACAGTTGCAAATAAGCGCATTAATAGCAAATTTAGTGAT
N Y K E T G T T V A N K R I N S K F S D
AGAGTCGATTTAATAGTAGGGGCTAAAGTAGCTGGTAGTACTGTGAATATAACTGATATT
R V D L I V G A K V A G S T V N I T D I
GTGATATATCCGGAAATTCATTCTTTTGTGGTGCACAAAGTAAATGGTAAATTATCTAAC
V I Y P E I H S F V V H K V N G K L S N
TCTCAGTCTATGTTAGATGGACAAACTGCTCCATTTATCAGTCAACCTGATAGAACTGCT
S Q S M L D G Q T A P F I S Q P D R T A
AAAACGTCTTATAATATAGGCTTAAGTGCAAACATAAAATCTGATGCTAAGATGGAGTAT
K T S Y N I G L S A N I K S D A K M E Y
GGTATCGGTTATGATTTTAATTCTGCAAGTAAATATACTGCACATCAAGGTACTTTAAAA
G I G Y D F N S A S K Y T A H Q G T L K
GTACGTGTAAACTTC
V R V N F -

Figure 16

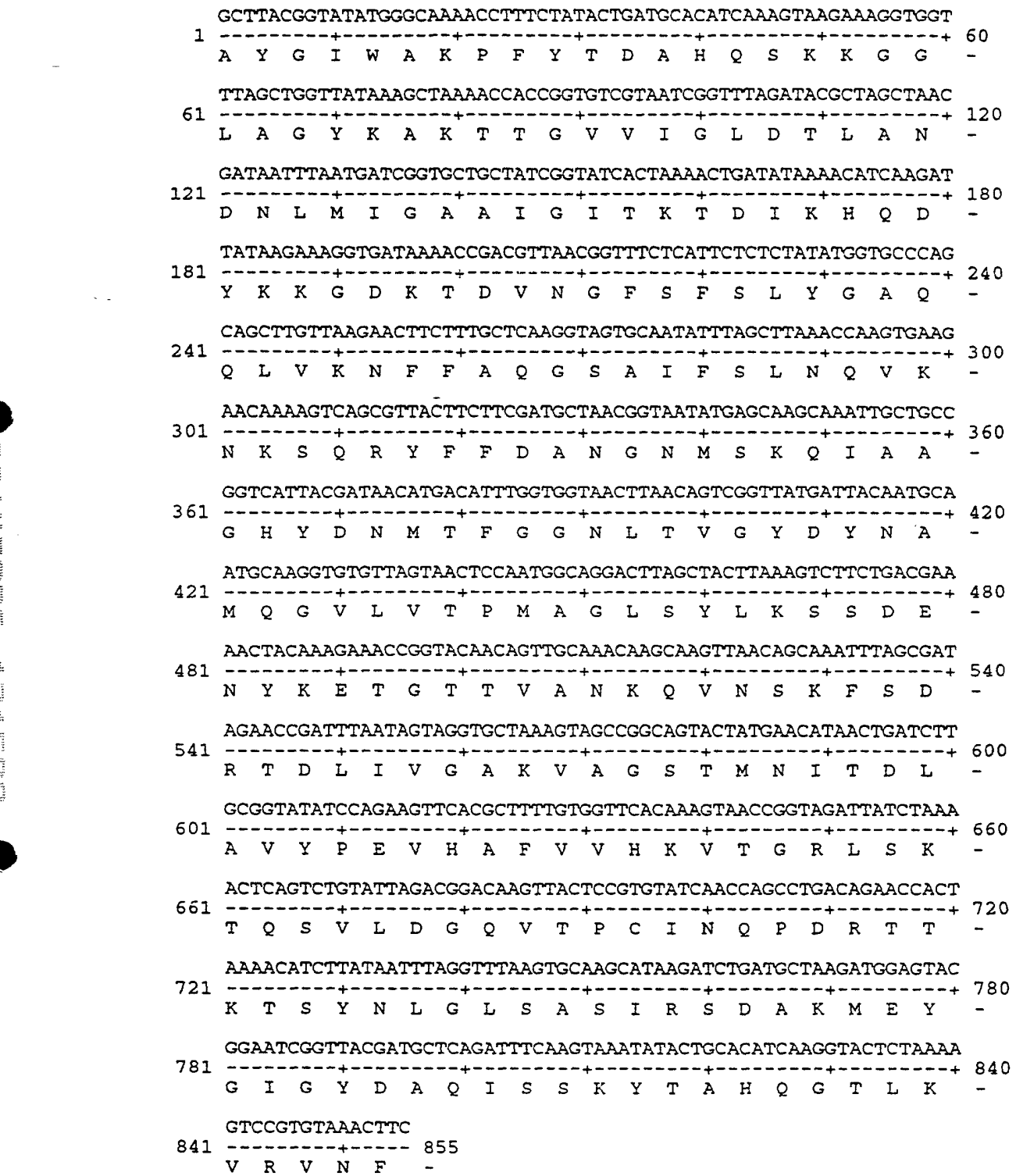


Figure 17

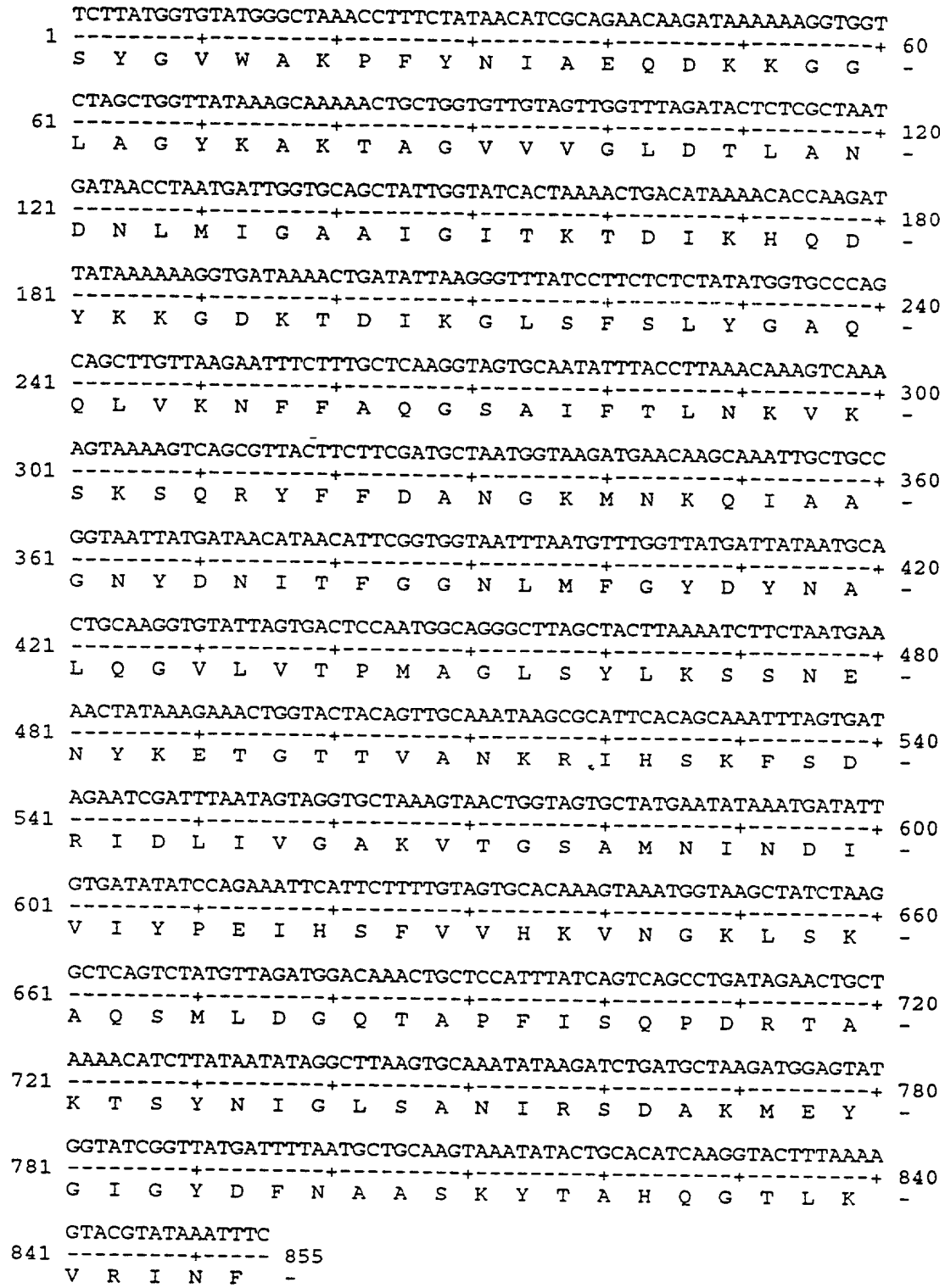

TCTTATGGTGTATGGGCTAAACCTTTCTATAACATCGCAGAACAAGATAAAAAAGGTGGT
S Y G V W A K P F Y N I A E Q D K K G G
CTAGCTGGTTATAAAGCAAAAACTGCTGGTGTTGTAGTTGGTTTAGATACTCTCGCTAAT
L A G Y K A K T A G V V V G L D T L A N
GATAACCTAATGATTGGTGCAGCTATTGGTATCACTAAAACTGACATAAAACACCAAGAT
D N L M I G A A I G I T K T D I K H Q D
TATAAAAAAGGTGATAAAACTGATATTAAGGGTTTATCCTTCTCTCTATATGGTGCCCAG
Y K K G D K T D I K G L S F S L Y G A Q
CAGCTTGTTAAGAATTTCTTTGCTCAAGGTAGTGCAATATTTACCTTAAACAAAGTCAAA
Q L V K N F F A Q G S A I F T L N K V K
AGTAAAAGTCAGCGTTACTTCTTCGATGCTAATGGTAAGATGAACAAGCAAATTGCTGCC
S K S Q R Y F F D A N G K M N K Q I A A
GGTAATTATGATAACATAACATTCGGTGGTAATTTAATGTTTGGTTATGATTATAATGCA
G N Y D N I T F G G N L M F G Y D Y N A
CTGCAAGGTGTATTAGTGACTCCAATGGCAGGGCTTAGCTACTTAAAATCTTCTAATGAA
L Q G V L V T P M A G L S Y L K S S N E
AACTATAAAGAAACTGGTACTACAGTTGCAAATAAGCGCATTCACAGCAAATTTAGTGAT
N Y K E T G T T V A N K R I H S K F S D
AGAATCGATTTAATAGTAGGTGCTAAAGTAACTGGTAGTGCTATGAATATAAATGATATT
R I D L I V G A K V T G S A M N I N D I
GTGATATATCCAGAAATTCATTCTTTTGTAGTGCACAAAGTAAATGGTAAGCTATCTAAG
V I Y P E I H S F V V H K V N G K L S K
GCTCAGTCTATGTTAGATGGACAAACTGCTCCATTTATCAGTCAGCCTGATAGAACTGCT
A Q S M L D G Q T A P F I S Q P D R T A
AAAACATCTTATAATATAGGCTTAAGTGCAAATATAAGATCTGATGCTAAGATGGAGTAT
K T S Y N I G L S A N I R S D A K M E Y
GGTATCGGTTATGATTTTAATGCTGCAAGTAAATATACTGCACATCAAGGTACTTTAAAA
G I G Y D F N A A S K Y T A H Q G T L K
GTACGTATAAATTTC
V R I N F -

Figure 18

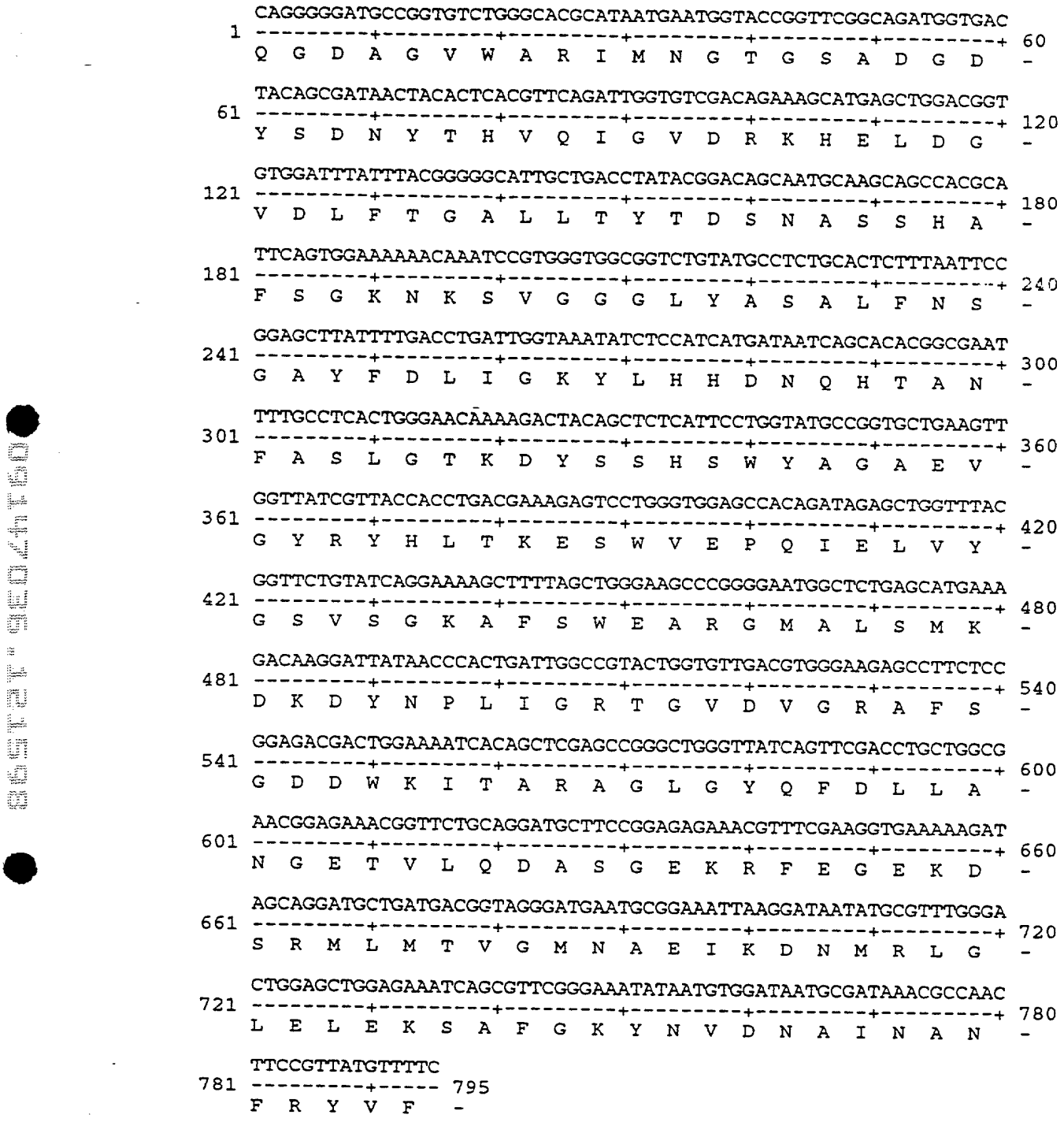

CAGGGGGATGCCGGTGTCTGGGCACGCATAATGAATGGTACCGGTTCGGCAGATGGTGAC
1
Q G D A G V W A R I M N G T G S A D G D
60
TACAGCGATAACTACACTCACGTTCAGATTGGTGTCGACAGAAAGCATGAGCTGGACGGT
61
Y S D N Y T H V Q I G V D R K H E L D G
120
GTGGATTTATTTACGGGGGCATTGCTGACCTATACGGACAGCAATGCAAGCAGCCACGCA
121
V D L F T G A L L T Y T D S N A S S H A
180
TTCAGTGGAAAAAACAAATCCGTGGGTGGCGGTCTGTATGCCTCTGCACTCTTTAATTCC
181
F S G K N K S V G G G L Y A S A L F N S
240
GGAGCTTATTTTGACCTGATTGGTAAATATCTCCATCATGATAATCAGCACACGGCGAAT
241
G A Y F D L I G K Y L H H D N Q H T A N
300
TTTGCCTCACTGGGAACAAAAGACTACAGCTCTCATTCCTGGTATGCCGGTGCTGAAGTT
301
F A S L G T K D Y S S H S W Y A G A E V
360
GGTTATCGTTACCACCTGACGAAAGAGTCCTGGGTGGAGCCACAGATAGAGCTGGTTTAC
361
G Y R Y H L T K E S W V E P Q I E L V Y
420
GGTTCTGTATCAGGAAAAGCTTTTAGCTGGGAAGCCCGGGGAATGGCTCTGAGCATGAAA
421
G S V S G K A F S W E A R G M A L S M K
480
GACAAGGATTATAACCCACTGATTGGCCGTACTGGTGTTGACGTGGGAAGAGCCTTCTCC
481
D K D Y N P L I G R T G V D V G R A F S
540
GGAGACGACTGGAAAATCACAGCTCGAGCCGGGCTGGGTTATCAGTTCGACCTGCTGGCG
541
G D D W K I T A R A G L G Y Q F D L L A
600
AACGGAGAAACGGTTCTGCAGGATGCTTCCGGAGAGAAACGTTTCGAAGGTGAAAAAGAT
601
N G E T V L Q D A S G E K R F E G E K D
660
AGCAGGATGCTGATGACGGTAGGGATGAATGCGGAAATTAAGGATAATATGCGTTTGGGA
661
S R M L M T V G M N A E I K D N M R L G
720
CTGGAGCTGGAGAAATCAGCGTTCGGGAAATATAATGTGGATAATGCGATAAACGCCAAC
721
L E L E K S A F G K Y N V D N A I N A N
780
TTCCGTTATGTTTTC
781
F R Y V F
795

Figure 19

```
      ACCCGTCAACTGTCCGGCCAGATCCACGCGGATATGGCGTCCGCCCAGATTAACGAAAGC
   1  ---------+---------+---------+---------+---------+---------+ 60
      T  R  Q  L  S  G  Q  I  H  A  D  M  A  S  A  Q  I  N  E  S  -

      CGTTATCTGCGCGATACCGCCACCGAGCGGTTGCGCCAGGCCGATGGCCGCCGCACCGCT
  61  ---------+---------+---------+---------+---------+---------+ 120
      R  Y  L  R  D  T  A  T  E  R  L  R  Q  A  D  G  R  R  T  A  -

      TCCGATATCAAAGCGGATGATAATGGCGCCTGGGCGAAATTGCTGGGCAACTGGGGGCAT
 121  ---------+---------+---------+---------+---------+---------+ 180
      S  D  I  K  A  D  D  N  G  A  W  A  K  L  L  G  N  W  G  H  -

      GCTTCCGGCAACGACAACGCTACCGGTTACCAGACATCCACCTATGGCGTGCTGTTGGGT
 181  ---------+---------+---------+---------+---------+---------+ 240
      A  S  G  N  D  N  A  T  G  Y  Q  T  S  T  Y  G  V  L  L  G  -

      CTGGACAGCGAACTGTTTGACGACGGCCGGCTGGGCGTGATGACCGGGTATACCCGCACG
 241  ---------+---------+---------+---------+---------+---------+ 300
      L  D  S  E  L  F  D  D  G  R  L  G  V  M  T  G  Y  T  R  T  -

      TCGCTGGTAGGCGGTCTACAGTCAGTAGTCCACAGCGACACTACACATCTGGGGCTGTAC
 301  ---------+---------+---------+---------+---------+---------+ 360
      S  L  V  G  G  L  Q  S  V  V  H  S  D  T  T  H  L  G  L  Y  -

      GGCGACAAACGCTTCGGCGCGTTGGCGCTGCCAGCGGGCGGCACCTATACCTGGCATCGC
 361  ---------+---------+---------+---------+---------+---------+ 420
      G  D  K  R  F  G  A  L  A  L  P  A  G  G  T  Y  T  W  H  R  -

      ATCGACACGTCGCGCTCGGTAAACTACGGCGCGCAGGCGGATCGCGAAAAGGCCCGCTAT
 421  ---------+---------+---------+---------+---------+---------+ 480
      I  D  T  S  R  S  V  N  Y  G  A  Q  A  D  R  E  K  A  R  Y  -

      AACGCGCGCACCGGTCAGCTGTTTATCGAAAGCGGCTACGATTGGAGCAACGACGTGGTC
 481  ---------+---------+---------+---------+---------+---------+ 540
      N  A  R  T  G  Q  L  F  I  E  S  G  Y  D  W  S  N  D  V  V  -

      AATCTTGAGCCGTTCGCCAACCTGGCGTACACCCACTATCGCAACGAGGGGATCAACGAG
 541  ---------+---------+---------+---------+---------+---------+ 600
      N  L  E  P  F  A  N  L  A  Y  T  H  Y  R  N  E  G  I  N  E  -

      CAAGGCGGGGCGGCGGCGCTGCGCGGCGATAAGCAAAGTCAGTCCGCCACCGCTTCGACG
 601  ---------+---------+---------+---------+---------+---------+ 660
      Q  G  G  A  A  A  L  R  G  D  K  Q  S  Q  S  A  T  A  S  T  -

      CTGGGCCTGCGCGCCGATACGCAATGGCAGACCGACAGCGTGGCGATCGCCCTGCCGGGC
 661  ---------+---------+---------+---------+---------+---------+ 720
      L  G  L  R  A  D  T  Q  W  Q  T  D  S  V  A  I  A  L  P  G  -

      GAGCTGGGTTGGCAACATCAGTACGGCAAGCTGGAGCGTAAAACACAGCTGATGTTCAAA
 721  ---------+---------+---------+---------+---------+---------+ 780
      E  L  G  W  Q  H  Q  Y  G  K  L  E  R  K  T  Q  L  M  F  K  -

      CGCAGCGATGTCGCGTTCGACGTGAACAGCGTCCCTGTTTCTCGCGATGGGGCCATTCTG
 781  ---------+---------+---------+---------+---------+---------+ 840
      R  S  D  V  A  F  D  V  N  S  V  P  V  S  R  D  G  A  I  L  -

      AAAGCGGGCGTCGATGTATCGATTAACAAAAACGTCGTCCTGTCCCTTGGGTACGGCGGG
 841  ---------+---------+---------+---------+---------+---------+ 900
      K  A  G  V  D  V  S  I  N  K  N  V  V  L  S  L  G  Y  G  G  -

      CAGCTGTCGTCCAACCACCAGGACAACAGCGTCAACGCCGGCCTGACCTGGCGGTTC
 901  ---------+---------+---------+---------+---------+------- 957
      Q  L  S  S  N  H  Q  D  N  S  V  N  A  G  L  T  W  R  F
```

Figure 20

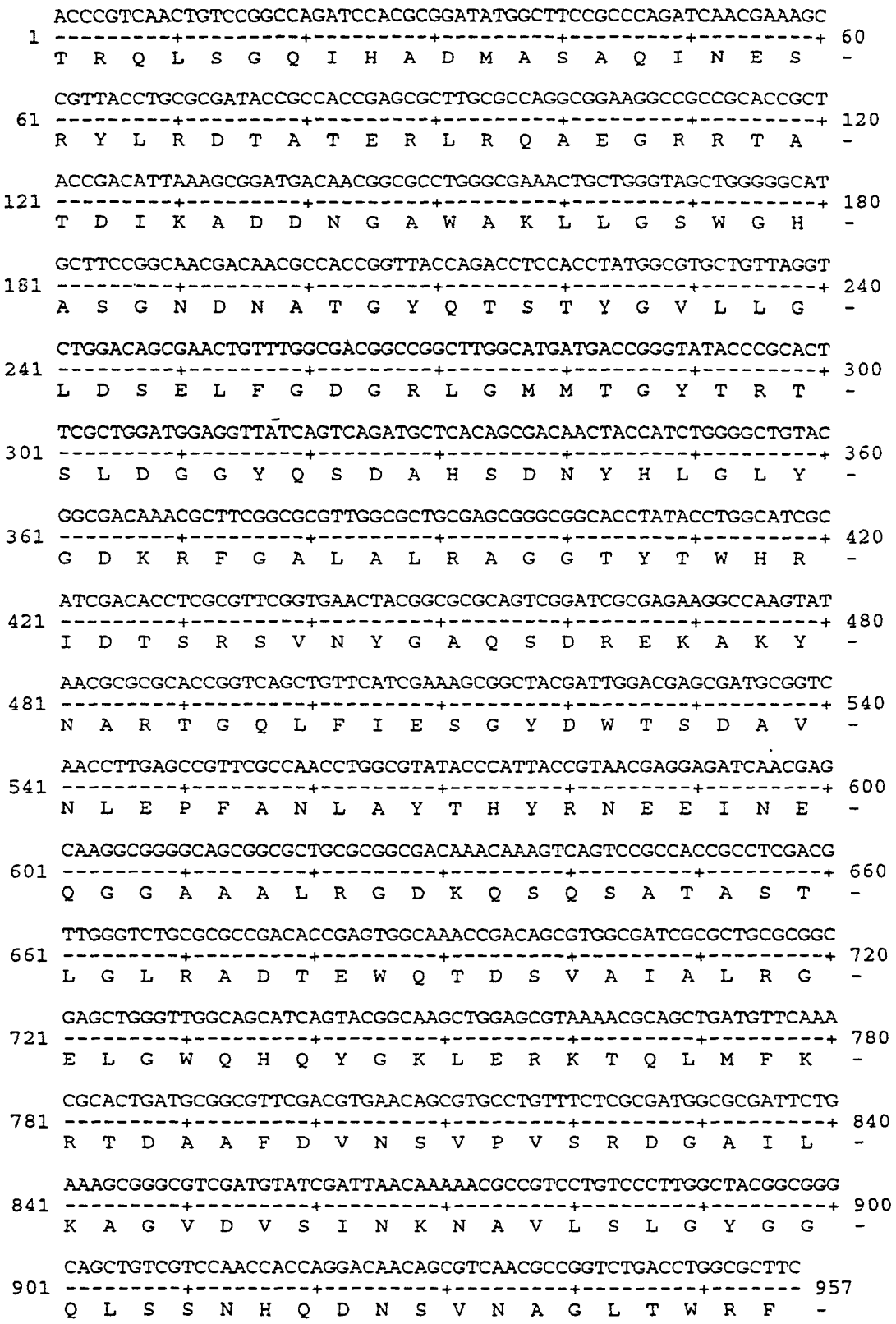


Figure 21

```
                TTCCGTCAGCTGTCGGGGCAAATCCATGCGGACATCGCGTCGGCGCTGGTGAACGACAGC
      1 ---------+---------+---------+---------+---------+---------+---------+ 60
                F  R  Q  L  S  G  Q  I  H  A  D  I  A  S  A  L  V  N  D  S  -

                CGCTACCTGCGTGAGGCGCTGAACGGGCGTCTGCGTCAGGCGGAAGGGCTGGCGAGCTCG
     61 ---------+---------+---------+---------+---------+---------+---------+ 120
                R  Y  L  R  E  A  L  N  G  R  L  R  Q  A  E  G  L  A  S  S  -

                TCGGCCATCAAGGCGGACGAGGACGGCGCCTGGGCGCAGCTGCTGGGAGCGTGGGACCAT
    121 ---------+---------+---------+---------+---------+---------+---------+ 180
                S  A  I  K  A  D  E  D  G  A  W  A  Q  L  L  G  A  W  D  H  -

                GCGTCGGGCGACGCCAACGCCACCGGCTATCAGGCCTCGACCTACGGGGTGCTGGTGGGG
    181 ---------+---------+---------+---------+---------+---------+---------+ 240
                A  S  G  D  A  N  A  T  G  Y  Q  A  S  T  Y  G  V  L  V  G  -

                CTGGACTCGGCGGCGGCGGCCGACTGGCGGCTGGGGGTGGCGACCGGCTACACCCGCACC
    241 ---------+---------+---------+---------+---------+---------+---------+ 300
                L  D  S  A  A  A  A  D  W  R  L  G  V  A  T  G  Y  T  R  T  -

                TCGCTGCACGGCGGGTATGGGTCGAAGGCGGACAGCGACAACTACCACCTGGCGGCGTAC
    301 ---------+---------+---------+---------+---------+---------+---------+ 360
                S  L  H  G  G  Y  G  S  K  A  D  S  D  N  Y  H  L  A  A  Y  -

                GGCGACAAGCAGTTCGGGGCGCTGGCGCTGCGGGGCGGGGCGGGCTACACCTGGCACCGC
    361 ---------+---------+---------+---------+---------+---------+---------+ 420
                G  D  K  Q  F  G  A  L  A  L  R  G  G  A  G  Y  T  W  H  R  -

                ATCGACACCAAGCGGTCGGTGAACTACGGGATGCAGTCGGACCGCGACACGGCGAAGTAC
    421 ---------+---------+---------+---------+---------+---------+---------+ 480
                I  D  T  K  R  S  V  N  Y  G  M  Q  S  D  R  D  T  A  K  Y  -

                AGCGCGCGCACCGAGCAGCTGTTCGCGGAAGCGGGCTACAGCGTGAAGGGCGAGTGGCTG
    481 ---------+---------+---------+---------+---------+---------+---------+ 540
                S  A  R  T  E  Q  L  F  A  E  A  G  Y  S  V  K  G  E  W  L  -

                AACCTGGAGCCGTTCGTCAACCTGGCGTACGTGAACTTTGAAAACAACGGCATCGCGGAA
    541 ---------+---------+---------+---------+---------+---------+---------+ 600
                N  L  E  P  F  V  N  L  A  Y  V  N  F  E  N  N  G  I  A  E  -

                AGCGGCGGCGCAGCGGCGCTGCGCGGCGACAAGCAGCACACCGACGCGACGGTGTCGACG
    601 ---------+---------+---------+---------+---------+---------+---------+ 660
                S  G  G  A  A  A  L  R  G  D  K  Q  H  T  D  A  T  V  S  T  -

                CTGGGACTGCGCGCGGACACTGAGTGGCAGGTGAGCCCGGGCACGACGGTGGCGCTGCGC
    661 ---------+---------+---------+---------+---------+---------+---------+ 720
                L  G  L  R  A  D  T  E  W  Q  V  S  P  G  T  T  V  A  L  R  -

                AGCGAGCTGGGGTGGCAACACCAGTACGGCGGGCTGGAGCGTGGCACCGGGCTGCGGTTC
    721 ---------+---------+---------+---------+---------+---------+---------+ 780
                S  E  L  G  W  Q  H  Q  Y  G  G  L  E  R  G  T  G  L  R  F  -

                AACGGCGGCAACGCGCCGTTCGTGGTGGACAGCGTGCCGGTGTCGCGCGACGGGATGGTG
    781 ---------+---------+---------+---------+---------+---------+---------+ 840
                N  G  G  N  A  P  F  V  V  D  S  V  P  V  S  R  D  G  M  V  -

                CTGAAGGCGGGTGCGGAAGTGGCGGTGAACGAGAACGCCTCGCTGTCGCTGGGCTACGGC
    841 ---------+---------+---------+---------+---------+---------+---------+ 900
                L  K  A  G  A  E  V  A  V  N  E  N  A  S  L  S  L  G  Y  G  -

                GGGCTGCTGTCGCAGAACCATCAGGACAACAGCGTCAACGCCGGCTTCACCTGGCGCTTC
    901 ---------+---------+---------+---------+---------+---------+---------+ 960
                G  L  L  S  Q  N  H  Q  D  N  S  V  N  A  G  F  T  W  R  F  -
```

Figure 22

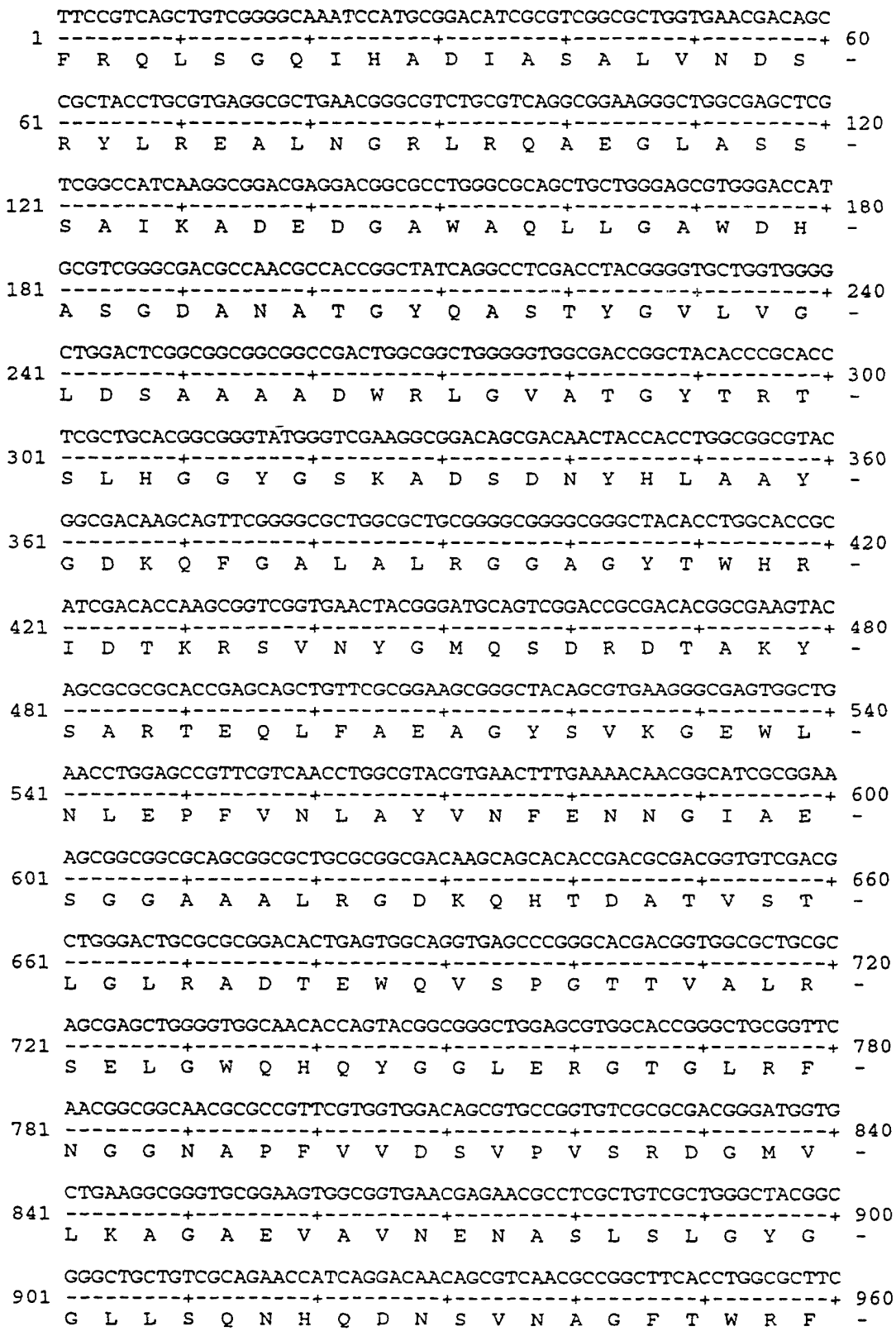


Figure 23

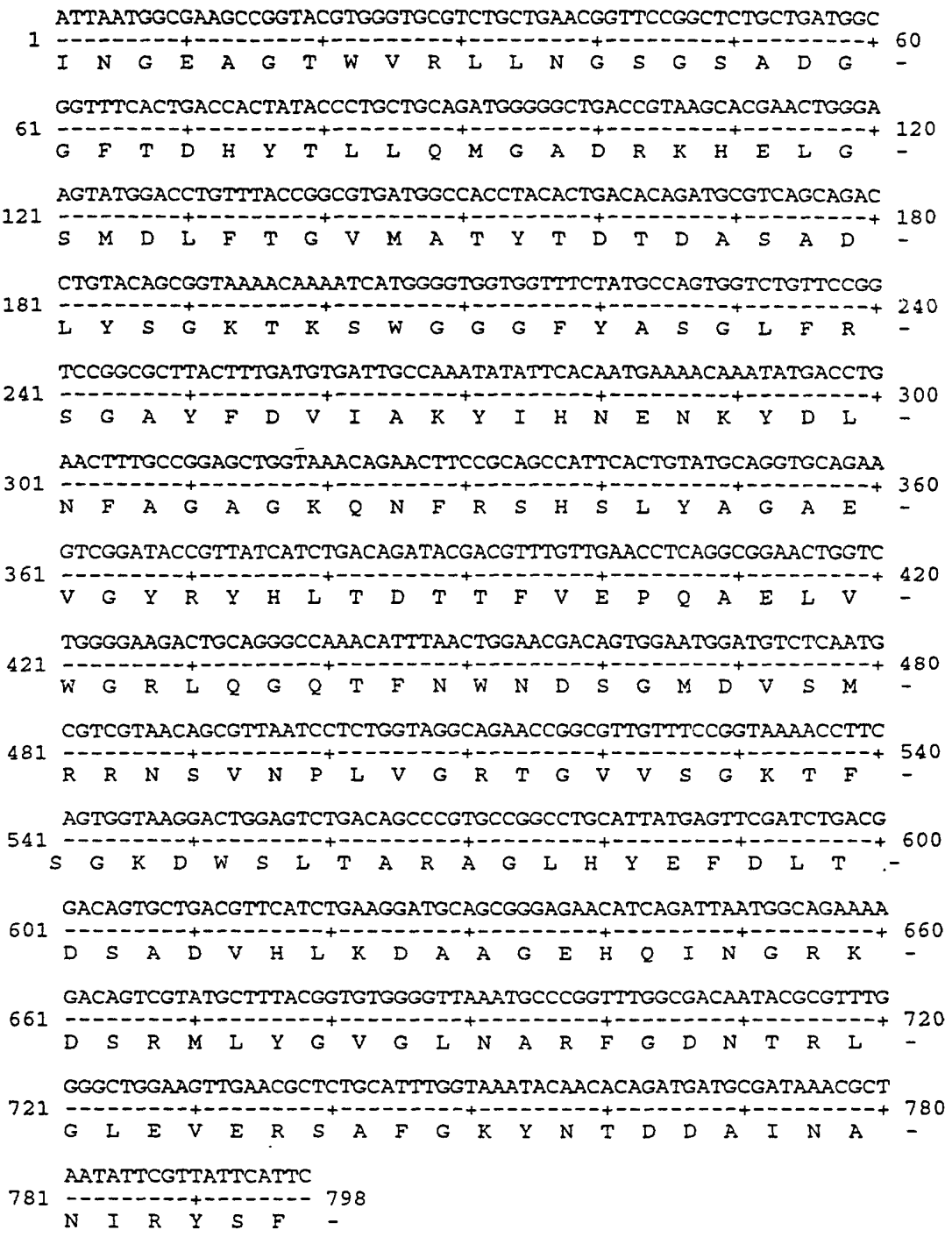
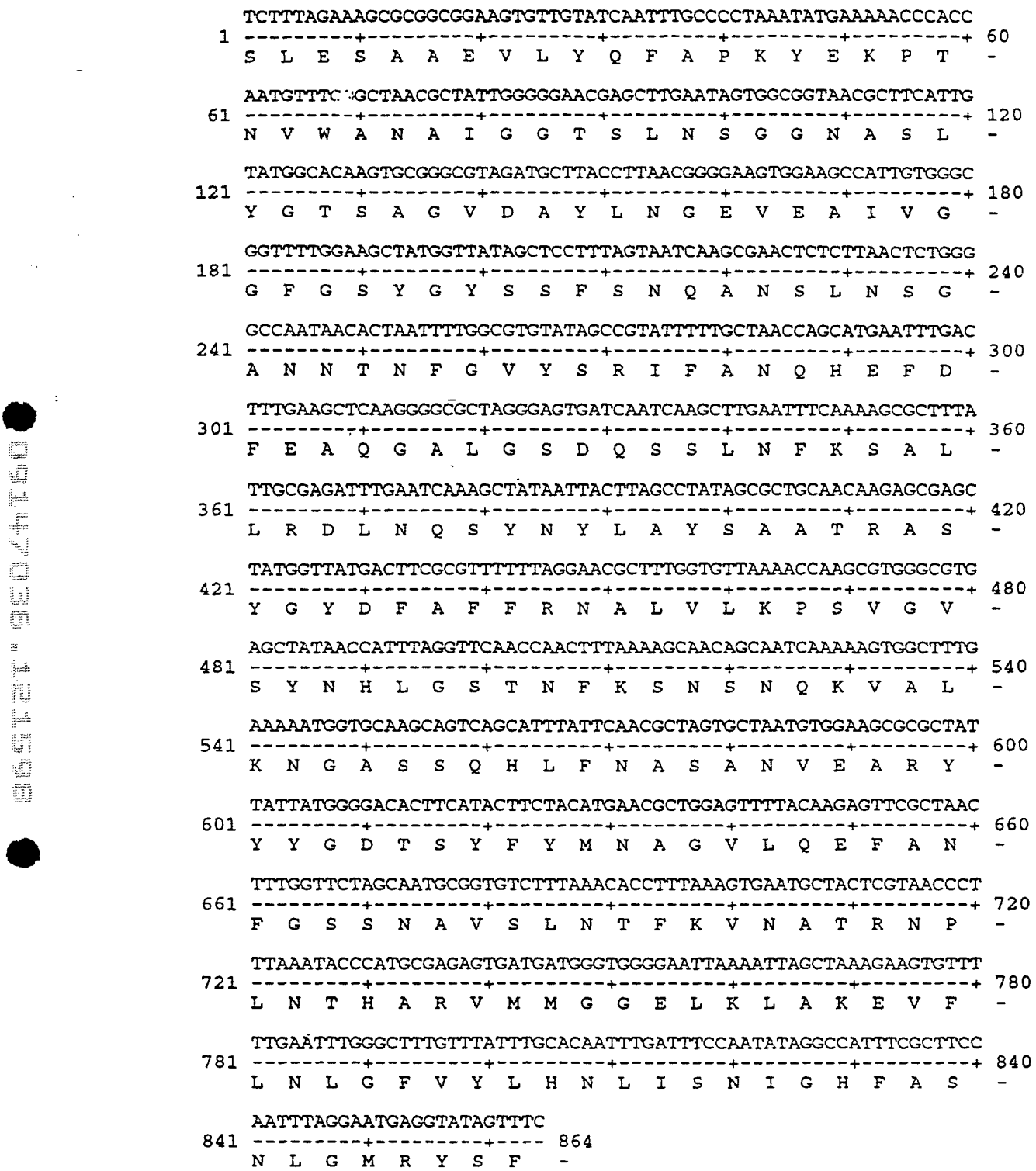

ATTAATGGCGAAGCCGGTACGTGGGTGCGTCTGCTGAACGGTTCCGGCTCTGCTGATGGC
1 60
I N G E A G T W V R L L N G S G S A D G -
GGTTTCACTGACCACTATACCCTGCTGCAGATGGGGGCTGACCGTAAGCACGAACTGGGA
61 120
G F T D H Y T L L Q M G A D R K H E L G -
AGTATGGACCTGTTTACCGGCGTGATGGCCACCTACACTGACACAGATGCGTCAGCAGAC
121 180
S M D L F T G V M A T Y T D T D A S A D -
CTGTACAGCGGTAAAACAAAATCATGGGGTGGTGGTTTCTATGCCAGTGGTCTGTTCCGG
181 240
L Y S G K T K S W G G G F Y A S G L F R -
TCCGGCGCTTACTTTGATGTGATTGCCAAATATATTCACAATGAAAACAAATATGACCTG
241 300
S G A Y F D V I A K Y I H N E N K Y D L -
AACTTTGCCGGAGCTGGTAAACAGAACTTCCGCAGCCATTCACTGTATGCAGGTGCAGAA
301 360
N F A G A G K Q N F R S H S L Y A G A E -
GTCGGATACCGTTATCATCTGACAGATACGACGTTTGTTGAACCTCAGGCGGAACTGGTC
361 420
V G Y R Y H L T D T T F V E P Q A E L V -
TGGGGAAGACTGCAGGGCCAAACATTTAACTGGAACGACAGTGGAATGGATGTCTCAATG
421 480
W G R L Q G Q T F N W N D S G M D V S M -
CGTCGTAACAGCGTTAATCCTCTGGTAGGCAGAACCGGCGTTGTTTCCGGTAAAACCTTC
481 540
R R N S V N P L V G R T G V V S G K T F -
AGTGGTAAGGACTGGAGTCTGACAGCCCGTGCCGGCCTGCATTATGAGTTCGATCTGACG
541 600
S G K D W S L T A R A G L H Y E F D L T -
GACAGTGCTGACGTTCATCTGAAGGATGCAGCGGGAGAACATCAGATTAATGGCAGAAAA
601 660
D S A D V H L K D A A G E H Q I N G R K -
GACAGTCGTATGCTTTACGGTGTGGGGTTAAATGCCCGGTTTGGCGACAATACGCGTTTG
661 720
D S R M L Y G V G L N A R F G D N T R L -
GGGCTGGAAGTTGAACGCTCTGCATTTGGTAAATACAACACAGATGATGCGATAAACGCT
721 780
G L E V E R S A F G K Y N T D D A I N A -
AATATTCGTTATTCATTC
781 798
N I R Y S F -

Figure 24


TCTTTAGAAAGCGCGGCGGAAGTGTTGTATCAATTTGCCCCTAAATATGAAAAACCCACC
1 60
S L E S A A E V L Y Q F A P K Y E K P T -
AATGTTTC GCTAACGCTATTGGGGGAACGAGCTTGAATAGTGGCGGTAACGCTTCATTG
61 120
N V W A N A I G G T S L N S G G N A S L -
TATGGCACAAGTGCGGGCGTAGATGCTTACCTTAACGGGGAAGTGGAAGCCATTGTGGGC
121 180
Y G T S A G V D A Y L N G E V E A I V G -
GGTTTTGGAAGCTATGGTTATAGCTCCTTTAGTAATCAAGCGAACTCTCTTAACTCTGGG
181 240
G F G S Y G Y S S F S N Q A N S L N S G -
GCCAATAACACTAATTTTGGCGTGTATAGCCGTATTTTTGCTAACCAGCATGAATTTGAC
241 300
A N N T N F G V Y S R I F A N Q H E F D -
TTTGAAGCTCAAGGGGCGCTAGGGAGTGATCAATCAAGCTTGAATTTCAAAAGCGCTTTA
301 360
F E A Q G A L G S D Q S S L N F K S A L -
TTGCGAGATTTGAATCAAAGCTATAATTACTTAGCCTATAGCGCTGCAACAAGAGCGAGC
361 420
L R D L N Q S Y N Y L A Y S A A T R A S -
TATGGTTATGACTTCGCGTTTTTTAGGAACGCTTTGGTGTTAAAACCAAGCGTGGGCGTG
421 480
Y G Y D F A F F R N A L V L K P S V G V -
AGCTATAACCATTTAGGTTCAACCAACTTTAAAAGCAACAGCAATCAAAAAGTGGCTTTG
481 540
S Y N H L G S T N F K S N S N Q K V A L -
AAAAATGGTGCAAGCAGTCAGCATTTATTCAACGCTAGTGCTAATGTGGAAGCGCGCTAT
541 600
K N G A S S Q H L F N A S A N V E A R Y -
TATTATGGGGACACTTCATACTTCTACATGAACGCTGGAGTTTTACAAGAGTTCGCTAAC
601 660
Y Y G D T S Y F Y M N A G V L Q E F A N -
TTTGGTTCTAGCAATGCGGTGTCTTTAAACACCTTTAAAGTGAATGCTACTCGTAACCCT
661 720
F G S S N A V S L N T F K V N A T R N P -
TTAAATACCCATGCGAGAGTGATGATGGGTGGGGAATTAAAATTAGCTAAAGAAGTGTTT
721 780
L N T H A R V M M G G E L K L A K E V F -
TTGAATTTGGGCTTTGTTTATTTGCACAATTTGATTTCCAATATAGGCCATTTCGCTTCC
781 840
L N L G F V Y L H N L I S N I G H F A S -
AATTTAGGAATGAGGTATAGTTTC
841 864
N L G M R Y S F -

N, M, M & O Docket No. _____ NIKAIDO, MARMELSTEIN, MURRAY & ORAM

Declaration For U.S. Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled (Insert Title) Export systems for recombinant proteins

the specification of which:

(Check one of blocks 1, 2 or 3. See note A on back of this page)

1. ☐ is attached hereto.
2. ☒ was filed on 15 March 1996 as International PCT Application Serial No. PCT/EP96/01130 and was amended on 06 July 1996 (if applicable).
3. ☐ was filed on _____ as U.S. Application Serial No. _____ and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application for which priority is claimed:

(List prior foreign applications. See note B on back of this page)	(Number)	(Country)	(Day/Month/Year Filed)	Priority Claimed
				☐ Yes ☐ No
				☐ Yes ☐ No
				☐ Yes ☐ No
				☐ Yes ☐ No

(See Note C on back of this page) ☐ See attached list for additional prior foreign applications

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT International filing date of this application:

(List prior U.S. Applications or PCT International applications designating the U.S.)	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

And I hereby appoint as principal attorneys David T. Nikaido, Reg. No. 22,663; Charles M. Marmelstein, Reg. No. 25,895, George B. Oram, Jr., Reg. No. 27,931; Robert B. Murray, Reg. No. 22,980; Martin S. Postman, Reg. No. 18,570; E. Marcia Ennis, Reg. No. 32,131; Michael G. Oliman, Reg. No. 19,114, Douglas H. Goldhush, Reg. No. 33,125; Kevin C. Brown, Reg. No. 32,402; Monica Chin Kitz, Reg. No. 36,105; Sharon N. Kinsner, Reg. No. 36,335, and John R. Fuisz, Reg. No. 37,227.

Please direct all communications to the following address: NIKAIDO, MARMELSTEIN, MURRAY & ORAM
Metropolitan Square
655 Fifteenth Street, N.W., Suite 330 - G Street Lobby
Washington, D.C. 20005-5701
(202) 638-5000 Fax: (202) 638-4810

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(See Note D on back of this page)

Full name of sole or first inventor Jochen Maurer

Inventor's signature _____ Date _____

Residence D-72070 Tübingen, Federal Republic of Germany

Citizenship German

Post Office Address Pfleghofstraße 10, D-72070 Tübingen, Germany

Full name of second joint inventor, if any Joachim Jose

Inventor's signature [signature] 14.09.92
Date

Residence D-72076 Tübingen, Federal Republic of Germany

Citizenship Germany

Post Office Address Hartmeyerstraße 48, D-72076 Tübingen, Germany

Full name of third joint inventor, if any Thomas F. Meyer

Inventor's signature
Date

Residence D-72076 Tübingen, Federal Republic of Germany

Citizenship Germany

Post Office Address Spemannstraße 30, D-72076 Tübingen, Germany

Full name of fourth joint inventor, if any

Inventor's signature
Date

Residence

Citizenship

Post Office Address

Full name of fifth joint inventor, if any

Inventor's signature
Date

Residence

Citizenship

Post Office Address

Full name of sixth joint inventor, if any

Inventor's signature
Date

Residence

Citizenship

Post Office Address

Full name of seventh joint inventor, if any

Inventor's signature
Date

Residence

Citizenship

Post Office Address

Full name of eighth joint inventor, if any

Inventor's signature
Date

Residence

Citizenship

Post Office Address

N, M, M & O Docket No. _____ NIKAIDO, MARMELSTEIN, MURRAY & ORAM

Declaration For U.S. Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
(Insert Title) Export systems for recombinant proteins
the specification of which

(Check one of blocks 1, 2 or 3. See note A on back of this page)

1. ☐ is attached hereto.
2. ☒ was filed on 15 March 1996 as International PCT Application Serial No. PCT/EP96/01130 and was amended on 06 July 1998 (if applicable)
3. ☐ was filed on _____ as U.S. Application Serial No. _____ and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application for which priority is claimed:

(List prior foreign applications. See note B on back of this page)

(Number)	(Country)	(Day/Month/Year Filed)	Priority Claimed
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No

(See Note C on back of this page) ☐ See attached list for additional prior foreign applications

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT International filing date of this application:

(List prior U.S. Applications or PCT International applications designating the U.S.)

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

And I hereby appoint as principal attorneys David T. Nikaido, Reg. No. 22,663; Charles M. Marmelstein, Reg. No. 25,895; George E. Oram, Jr., Reg. No. 27,931; Robert B. Murray, Reg. No. 22,980; Martin S. Postman, Reg. No. 18,570; E. Marcie Emas, Reg. No. 32,131; Michael G. Gilman, Reg. No. 19,114, Douglas H. Goldhush, Reg. No. 33,125, Kevin C. Brown, Reg. No. 32,402; Monica Chin Kitts, Reg. No. 36,105; Sharon N. Klesner, Reg. No. 36,335, and John R. Fulsz, Reg. No. 37,327.

Please direct all communications to the following address: NIKAIDO, MARMELSTEIN, MURRAY & ORAM
Metropolitan Square
655 Fifteenth Street, N.W., Suite 330 - G Street Lobby
Washington, D.C. 20005-5701
(202) 638-5000 Fax: (202) 638-4810

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(See Note D on back of this page)

Full name of sole or first inventor Jochen Maurer
Inventor's signature _____ Date DEC
Residence D-72070 Tübingen, Federal Republic of Germany
Citizenship German
Post Office Address Pfleghofstraße 10, D-72070 Tübingen, German

Full name of second joint inventor, if any Joachim Jose

Inventor's signature X Date

Residence [redacted] Republic of Germany

Citizenship Germany

Post Office Address Hartmeyerstraße 48, D-72076 Tübingen, Germany

Full name of third joint inventor, if any Thomas F. Meyer

Inventor's signature X 14. Sept. 98 Date

Residence D-72076 Tübingen, Federal Republic of Germany

Citizenship Germany

Post Office Address Spemannstraße 30, D-72076 Tübingen, Germany

Full name of fourth joint inventor, if any

Inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of fifth joint inventor, if any

Inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of sixth joint inventor, if any

Inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of seventh joint inventor, if any

Inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of eighth joint inventor, if any

Inventor's signature Date

Residence

Citizenship

Post Office Address

NOTES

A. 1. For declaration to be filed with original U.S. (Non-PCT) Application Papers, check box 1.

2. For declaration to be filed in U.S. National Phase of PCT Application (either with original national phase entry papers or subsequent to expiration of 20 or 30 month term), check box 2, and complete information.

3. For declaration to be filed after original U.S. (Non-PCT) Application filing date, check box 3, and complete information.

B. Please list all non-convention foreign applications relating to the invention (and check block "no"), as well as all convention (priority) applications.

C. If more than 4 prior foreign applications, please check this box and attach a sheet listing the remaining prior foreign applications.

D. For the Inventor's "Residence", only the city and state is necessary, however the "Post Office Address" must be an address acceptable by a Post Office for delivery of mail.

N, M, M & O Docket No. _____ NIKAIDO, MARMELSTEIN, MURRAY & ORAM

Declaration For U.S. Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

(Insert Title) Export systems for recombinant proteins

the specification of which

(Check one of blocks 1, 2 or 3. See note A on back of this page)

1. ☐ is attached hereto
2. ☒ was filed on 15 March 1996 as International PCT Application Serial No. PCT/EP96/01130 and was amended on 06 July 1998 (if applicable)
3. ☐ was filed on _____ as U.S. Application Serial No. _____ and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a)

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application for which priority is claimed.

(List prior foreign applications See note B on back of this page)	(Number)	(Country)	(Day/Month/Year Filed)	Priority Claimed
	(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes ☐ No
	(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes ☐ No
	(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes ☐ No

(See Note C on back of this page) ☐ See attached list for additional prior foreign applications

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT International filing date of this application:

(List prior U.S. Applications or PCT International applications designating the U.S.)	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

And I hereby appoint as principal attorneys David T. Nikaido, Reg. No. 22,663; Charles M. Marmelstein, Reg. No. 25,895; George R. Oram, Jr., Reg. No. 27,931; Robert B. Murray, Reg. No. 22,980, Martin S. Postman, Reg. No. 18,570; E. Marcie Emas, Reg. No. 32,131; Michael G. Gilman, Reg. No. 19,114, Douglas H. Goldhush, Reg. No. 33,125, Kevin C. Brown, Reg. No. 32,402; Monica Chin Kitts, Reg. No. 36,105; Sharon N. Kleaner, Reg. No. 36,335, and John R. Fulez, Reg. No. 37,327.

Please direct all communications to the following address: NIKAIDO, MARMELSTEIN, MURRAY & ORAM
Metropolitan Square
655 Fifteenth Street, N.W., Suite 330 - G Street Lobby
Washington, D.C. 20005-5701
(202) 638-5000 Fax: (202) 638-4810

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(See Note D on back of this page)

Full name of sole or first inventor Jochen Maurer

Inventor's signature X _____ Date _____

Residence D-72070 Tübingen, Federal Republic of Germany

Citizenship German

Post Office Address Pfleghofstraße 10, D-72070 Tübingen, Germany

Full name of second joint inventor, if any Joachim Jose

Inventor's signature X Date

Residence D-72076 Tübingen, Federal Republic of Germany DEX

Citizenship Germany

Post Office Address Hartmeyerstraße 48, D-72076 Tübingen, Germany

Full name of third joint inventor, if any Thomas F. Meyer

Inventor's signature X Date

Residence D-72076 Tübingen, Federal Republic of Germany DEX

Citizenship Germany

Post Office Address Sremarnstraße 30, D-72076 Tübingen, Germany

Full name of fourth joint inventor, if any

Inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of fifth joint inventor, if any

Inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of sixth joint inventor, if any

Inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of seventh joint inventor, if any

Inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of eighth joint inventor, if any

Inventor's signature Date

Residence

Citizenship

Post Office Address